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ARGININE METABOLISM AND CITRULLINE SYNTHESIS IN RAT SKELETAL MUSCLE

BY

DELORES SUSANNE PETERS

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

in

ANIMAL BIOCHEMISTRY

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA

SPRING 1994

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ABSTRACT

The contribution to citrulline biosynthesis from arginine deiminases, urea cycle enzymes or nitric oxide synthases (NOS) in rat skeletal muscle was investigated. First, modifications to several methods were made. It was verified that measuring total protein degradation by using proteins prelabelled with tritiated phenylalanine was sensitive and repeatable. An HPLC method for measuring citrulline was improved. A correction factor for the amount of citrulline lost during acid hydrolysis (47% in 24 hr) of proteins was determined. A cation exchange chromatography method was modified to separate arginine, citrulline and urea. Using these methods, citrulline production in skeletal muscle was measured. Incubated epitrochlearis or gastrocnemius muscle strips released 0.110-0.270 nmol citrulline/mg tissue/3 hr. This is an overestimate (27-32%) of synthesis since the amount of intracellular free citrulline decreased during incubation. This was not significantly affected by pretreating rats with endotoxin or by incubation with a NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) indicating that citrulline release can be compared between treatments. Citrulline release was not significantly affected by *in vivo* endotoxin injection, trauma or by *in vitro* changes in arginine, ornithine, or cycloheximide concentrations. The tyrosine:citrulline ratio was 2.8:1 in the incubation media, and ≥ 48:1 in muscle protein. These results are consistent with Ca^{++}-dependent NOS but inconsistent with arginine deiminase, Ca^{++}-independent NOS or urea cycle synthesis of citrulline. When intact muscles were incubated with tracer amounts of [*guanido*-^{14}C]arginine, [^{14}C]urea and [^{14}C]citrulline were synthesized (0.027 ± 0.003 and 0.074 ± 0.005 nmol/mg tissue/3 hr, respectively). A long incubation period (9 hr) and a high dose of L-NAME (2 mM) was required to inhibit synthesis of labelled citrulline (37% of control values). As well, [^{14}C]citrulline synthesis (0.261 ± 0.075 nmol/g tissue/min) and the inhibitory efficacy of L-NMMA and L-NAME were measured in muscle cytosol. Maximal inhibition occurred at 300 μM L-NMMA or 100 μM L-NAME. It was concluded that arginine is a metabolic precursor for citrulline synthesis in skeletal muscle by the activity of nitric oxide synthase, although this enzyme is not the only source of citrulline synthesis. Furthermore, it has been demonstrated that arginase is active in muscle tissue.

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LIST OF ABBREVIATIONS

A.A.	working amino acid standard for HPLC
AGPA	L-α-amino β-guanidino proprionic acid
AMP	adenosine 5'-monophosphate
arg	arginine
asp	aspartic acid
cit	citrulline
DNA	deoxyribonucleic acid
EGTA	ethylenediaminetetraacetate
glu	glutamic acid
gly	glycine
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
his	histidine
HPLC	High Performance Liquid Chromatography
IGF_1 and IGF_2	insulin like growth factor one and two
I.S.	working internal standard solution for HPLC
KRB	Krebs Ringer Buffer
L-NMMA	N^G-L-mono methyl arginine
L-NAME	N^G-L-nitro-arginine methyl ester
NADPH	nicotinamide-adenine dinucleotide phosphate
NOS	nitric oxide synthase
iNOS	inducible nitric oxide synthase
cNOS	constitutive Nitric Oxide Synthase
NMDA	N-methyl-D-aspartate
PMSF	phenylmethylsulphonyl flouride

RNA	ribonucleic acid
mRNA	messenger RNA
S.E.M.	standard error of the mean
ser	serine
STD	standard
thr	threonine
TCA	trichloroacetic acid

I. GENERAL INTRODUCTION

Since the skeletal muscle is a large organ of the body, approximately 40% by weight, it forms a large part of whole body amino acid metabolism. Traditionally, neither citrulline, nor its precursor arginine were thought to be synthesized or metabolized in skeletal muscle. The enzyme nitric oxide synthase, which catalyzes the formation of nitric oxide and citrulline from arginine, has recently been discovered to be active in skeletal muscle cytosol (1). This raises the question of how citrulline is synthesized in muscle, in what quantity, what are its pathways of synthesis, and are any other sources of citrulline present in skeletal muscle.

This thesis explores the sources of citrulline in skeletal muscle. In this chapter the literature is reviewed, outlining current knowledge of citrulline sources be they from protein degradation or from the activity of different enzymes. The research plan, with specific hypotheses and objectives, is presented in Chapter II. Chapters' III and IV describe verification of a method for measuring protein degradation and modifications to methods for measuring citrulline, respectively. The fifth chapter presents results of a series of experiments measuring citrulline release and synthesis in intact muscle or muscle cytosol. The final chapter (VI) discusses areas for future research and the possible physiological role of arginase and nitric oxide synthase in skeletal muscle.

SKELETAL MUSCLE AMINO ACID METABOLISM

Protein Turnover

The skeletal muscle intracellular amino acid pool, which includes citrulline, has three sources, absorption from the blood, *de novo* synthesis (see below) and amino acids released from degraded proteins. These amino acids can be used for protein synthesis, metabolism (see below) or released into the circulation. Therefore, one determinant of free amino acid pool size in in tissue protein skeletal muscle is the rate of protein synthesis and degradation. The balance between synthesis and degradation rates determines protein turnover. Any change in the relative rates of protein synthesis and degradation will alter the total amount of protein in the tissue. In a healthy animal that is not growing, the rate of synthesis equals the rate of degradation, while in a growing animal synthesis must be greater than degradation. Protein synthesis is controlled in three ways, by increasing the synthesis rate of mRNA, decreasing the rate of mRNA degradation, or by increasing the rate of translation from each mRNA (2). For instance, insulin increases the rate of translation (3), while glucocorticoids lower the rate of translation. Net protein degradation is a normal response to fasting (even as short as overnight) or to starvation. The released amino acids can be used for protein synthesis, oxidation or gluconeogenesis (4). The regulation and mechanisms of protein degradation are not understood as well as those for synthesis. Currently we know of lysosomal, calcium dependent and ATP-ubiquitin dependent pathways of protein degradation (5,6,7). Since blocking these pathways does not eliminate all protein degradation, other degradation pathways may also be involved (7,8). Hormonal regulation of protein degradation exists, for instance insulin, IGF-1 and IGF-2 inhibit degradation (reviewed in 3). Relative rates of protein turnover (the sum of protein synthesis and degradation) will affect the amino acid pool size and composition.

Measuring relative rates of turnover in skeletal muscle would allow the calculation of how much citrulline flux in the amino acid pool is due to protein synthesis or degradation [degradation or synthesis (nmole phe/mg/3 hr) multiplied by cit:phe ratio in tissue protein]. Commonly used methods of measuring protein degradation in skeletal muscle are either simultaneous measurement of protein synthesis and net protein degradation (9) or by blocking protein synthesis and measuring total degradation (see Chapter 3). The first method, indirect, incorporates errors of each measurement into the calculation of total degradation and the second method introduces a potential toxin into the system (10). Wu and Thompson (11) used the release of radioactivity from prelabelled proteins as a direct measure of protein degradation but this method has not been well characterized. In short there are difficulties in measuring protein degradation and consequently its contribution to the intracellular amino acid pool. In contrast, protein synthesis is easily measured by incorporation of radiolabelled amino acids (3), so it is possible to estimate this drain from the amino acid pool.

Amino Acid Synthesis

Individual amino acid synthesis or catabolism will also alter the composition and size of the intracellular amino acid pool. Skeletal muscle is capable of synthesizing the amino acids glutamine (12), glutamate, alanine, aspartate and asparagine (2). Alanine and glutamine are the major amino acids released from the muscle (2,4). Alanine is a major gluconeogenic precursor transported to the liver and used for glucose synthesis, and glutamine carries amino groups released in metabolism to the kidney where they are important in acid base balance. Glutamine is also a gluconeogenic precursor (2).

Amino Acid Catabolism

All of the above amino acids plus the branched chain amino acids, leucine, isoleucine and valine can be metabolized in muscle. The carbon skeletons may be completely oxidized in the muscle or exported. The amino groups are either released or added to glutamate to become glutamine, which is exported. While blood plasma levels of branched chain amino acids are known to be regulated by muscle metabolism (2), the regulation of internal muscle oxidation of these amino acids is not known. According to current dogma, neither arginine or citrulline are metabolized or synthesized in skeletal muscle. A review of the literature reveals only three reports of skeletal muscle metabolism of citrulline and arginine. The first mention is in Pardridge *et al.* (13) who, in 1982, showed the disappearance of arginine and appearance of citrulline and ornithine in the media of cultured muscle cells originating from adult rat myoblasts. Arginine fell from approximately 325 to 50 μM over 50 hr incubation while ornithine increased from approximately 25 to 225 μM and citrulline increased from 0 to approximately 100 μM during the same time. The authors suggest that the data provides evidence for arginase but do not comment on the source of citrulline. Arginine metabolism in wounded muscle was reported by Albina *et al.* (14) who showed the appearance of a labelled metabolite (1.5 pmol/min/mg tissue) from [*guanido*-^{14}C]arginine. In the method cited by Albina *et al.* (14), Ruegg and Russell (15) claim that the labelled metabolite is urea, but do not test the possibility that it may be citrulline. Therefore it is uncertain whether the labelled metabolite is urea as Albina *et al.* (14) claim, citrulline formed via nitric oxide synthase activity, or a mixture of both. Salter *et al.* (1) measured nitric oxide synthase activity in rat skeletal muscle cytosol, demonstrating the formation of citrulline and metabolism of arginine.

ARGININE AND CITRULLINE

Arginine, a basic amino acid (Fig. 1.1), is considered conditionally-essential. It is not essential for healthy adults in that the body can synthesize all the arginine required, while growing or traumatized animals have been reported to have a dietary requirement (16,17). Arginine is required in the synthesis of many proteins, is a part of the urea cycle, is a precursor of creatine (an energy phosphagen)(18) and polyamines via ornithine (17) and is inter-converted to glutamate, proline and ornithine (17). With the discovery of nitric oxide synthase, it is now known that arginine is also the precursor for the bioregulatory molecule, nitric oxide.

Citrulline is a mono-amino, mono-carboxylic (Fig. 1.1), nonessential amino acid. As well as being synthesized in the body, citrulline can also be absorbed from dietary sources (16). It has a role in the urea cycle, is a by-product of nitric oxide synthesis and can be synthesized into arginine (see below). Citrulline has been found in proteins in hair follicles, keratin, medullary proteins of hair and quills (20) and muscle (21). Since citrulline is not coded for by DNA, it must be post-translationally modified from arginine if it appears in proteins.

Three metabolic pathways link arginine and citrulline; the nitric oxide synthases, the urea cycle enzymes and arginine deiminases. Each pathway, including location in the body, is discussed below.

NITRIC OXIDE SYNTHASE (E.C.1.14.23)

It is now well established (reviewed in 21-26) that mammalian cells have the capacity to synthesize nitric oxide and that it acts as an important bioregulatory molecule. Nitric oxide maintains a vasodilatory tone, is a neurotransmitter, and when produced in sufficient quantities inhibits iron containing enzymes like mitochondrial respiratory enzymes as well as DNA reductase, causing cell

death. Nitric oxide and citrulline are synthesized from arginine and molecular oxygen (Fig 1.2). Current research continues to identify all the intermediates of the reaction. The several isozymes of the enzyme can be divided into two major groups, a Ca^{++}-dependent constitutive isoform and a Ca^{++}-independent inducible isoform (23-25). The inducible isoform is synthesized *de novo* after exposure of cells to bacterial endotoxin or to pro-inflammatory cytokines. The prototypic form is found in activated, but not resting macrophages, and it has also been found in inflammatory and cytokine stimulated neutrophils, vascular smooth muscle (27), hepatocytes, and carcinoma cells. The constitutive isoform was first discovered in endothelial cells (26) and is found also in the central nervous system, some peripheral neurons, platelets, mast cells, cardiac muscle (28), skeletal muscle (1) and β cells of the pancreatic islets (24). If stimulated with endotoxin or cytokines both cardiac muscle (28) and endothelial cells (24) can produce the inducible form of NOS, as well as cNOS. There are two major distinguishing features between the two isoforms, the sensitivity to blockers of protein synthesis and sensitivity to calcium (25). The constitutive isoform is not sensitive to protein synthesis blockers. The inducible isoform is synthesized after the cells are stimulated with cytokines. If protein synthesis blockers are applied with or soon after the cytokines, synthesis of iNOS is prevented, but once the enzyme has been synthesized protein synthesis blockers have no effect. The constitutive isoform, but not the inducible isoform, requires physiological levels of calcium/calmodulin for activity. cNOS is activated by the increase in cytosolic calcium via acetylcholine in endothelial cells or by glutamate stimulation of NMDA receptors, which open calcium channels in neuronal tissue (25). The constitutive isoform has recently been divided into two subgroups (22), the endothelial and the central nervous type. Though they have the same cofactor requirements, they have different molecular weights (22) and the endothelial isoform may be membrane associated, while the central nervous isoform is found only in the cytosol (22).

UREA CYCLE ENZYMES

The urea cycle, as first proposed by Krebs and Henseleit in 1932, metabolizes ammonia into urea (reviewed in 29,30). Bicarbonate ion and ammonia are synthesized into carbamoyl phosphate (Fig. 1.3) which is joined to ornithine to form citrulline. The citrulline is combined with aspartate (source of the other nitrogen) to form argininosuccinate, a short-lived intermediate. Argininosuccinate is cleaved into arginine and fumarate. The arginine is cleaved into urea and ornithine, while the fumarate cycles through part of the TCA cycle to oxaloacetate where an amino group is added to form aspartate. Regulation of the entire cycle is not completely clear. The rate-limiting step appears to be carbamoyl phosphate synthetase (30) regulated by ammonia availability, including muscle amino acid degradation. Ornithine transcarbamoylase is inhibited by a high level of ornithine (29). Argininosuccinate synthase is inhibited by its products: AMP, inorganic phosphorus and argininosuccinate.

Liver

The complete urea cycle occurs only in the liver. In normal physiological conditions there is no net synthesis of either arginine or citrulline (31). The low activity of the y^+ carrier prevents large amounts of arginine from being transported into hepatocytes (30).

Kidney

All the enzymes of the urea cycle are present in the kidney, but not in the ratio required to have a functional urea cycle (29). The rat kidney is capable of transforming large amounts of citrulline into arginine (16) (60.5 ± 22.15 nmol/min/mg dry wt) using the enzymes argininosuccinate synthetase and argininosuccinate lyase. The kidney appears to be the major source of arginine synthesis in the body (17). The kidney's ability to synthesize arginine from citrulline explains why, in growing animals, citrulline can replace arginine in the diet (17).

Small Intestine

Citrulline is synthesized in the small intestine from glutamine as demonstrated by Windmueller and Spaeth (33). Several enzymes are involved in this pathway, with the final step being ornithine transcarbamoylmase. There is no further metabolism of citrulline in the intestine. Arginase in small

amounts (2-5% of amounts in liver) has been described throughout the gastrointestinal tract (32,34) metabolizing 40% of absorbed arginine into ornithine and its metabolites, including citrulline.

Vasculature, Circulating Cells and Immune Cells

Several enzymes of the urea cycle are present in the circulatory and immune systems. Red blood cells contain large amounts of arginase (29) and serum has low levels of argininosuccinate activity (29). Citrulline is converted to arginine in vascular endothelial cells. The mechanism may be via the urea cycle enzymes' argininosuccinate synthetase and argininosuccinate lyase since argininosuccinate is also present (35,36). The same pathway exists in peritoneal macrophages (37).

Brain

Moderate amounts of argininosuccinate synthetase and argininosuccinate lyase are present in the brain. This would permit the brain to synthesize arginine from citrulline (26), though it is not a major contributor to the body pool (38).

Muscle

Arginase activity has been measured in cardiac muscle (34) skeletal muscle (19,34) and gastrointestinal smooth muscle (34), metabolizing arginine into ornithine and urea. The activity in these tissues is at least five fold less than that measured in liver. Argininosuccinate lyase has also been found in muscle (29). No evidence has been found for other urea cycle enzymes in muscle.

ARGININE DEIMINASES (E.C.3.5.3)

The arginine deiminases metabolize arginine into citrulline and ammonia (Fig. 1.4) (39,40). There are two enzymes, differentiated by substrate (40) and amino acid composition (41). Peptidylarginine deiminase (E.C.3.5.3.15), which only metabolizes arginine bound in peptide bonds, is found in mammalian hair follicles, skeletal muscle, brain, salivary glands, pancreas, uterus and many other tissues at lower levels (42). Citrulline residues have been found in keratin, medullary proteins of hair and quills, epidermis (140-190 μmol/g) and at a much lower level (1.4 μmol/g) in rabbit skeletal muscle (23), despite the high level of enzyme activity in muscle (42). This enzyme requires calcium, though its regulation is unknown. The second enzyme, arginine deiminase (E.C.3.5.3.6), can only metabolize free arginine (43). It has been described in prokaryotes and single cell eukaryotes (39,44), where it forms part of the pathway providing one ATP from the metabolism of arginine into ornithine, ammonia and carbon dioxide (39). This enzyme is inhibited by glucose and by ATP, and is stimulated by energy depletion (39). It requires no cofactors or metal ions (43).

MEASURING ARGININE, CITRULLINE AND UREA

One way to measure the activity of the enzymes of arginine metabolism is to measure the appearance of their products. Free amino acids can be measured by reverse phase HPLC. A standard method based on the method of Jones and Gilligan (45) has been prepared for the Animal Science Laboratory, University of Alberta. This method allows measurement of 21 amino acids including arginine, citrulline, ornithine and tyrosine, but not urea. Urea, a product of arginase, can be measured by two methods, a colourimetric method which reacts with the ureido group, and by an enzymatic method in which urease hydrolyses urea and subsequently the ammonium ion produced is measured. Difficulties exist with both systems. The colourimetric reagent also reacts with the ureido group of citrulline (46), so cannot be used where both citrulline and urea are expected. The urease system is frequently contaminated with arginase (cited in 15), which will generate urea in the presence of arginine, and hence cannot be used where arginine is present. Using radiolabelled substrate is a sensitive way to measure small amounts of urea or citrulline synthesis. It would be necessary to separate urea from citrulline and both from the labelled substrate, arginine, as Gopalakrishna and Nagarajan (47) have done in acidic intracellular tissue fraction. If this method could be applied to physiological buffers

(pH 7.4) containing electrolytes, it would be a sensitive and specific method for measuring both citrulline and urea.

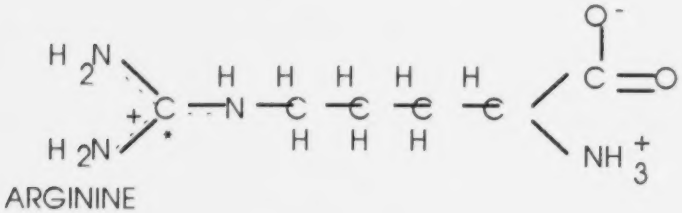
SUMMARY

Of the pathways of arginine metabolism to citrulline in the body, only nitric oxide synthase (1) has been suggested to be present in skeletal muscle. The urea cycle enzymes, present in a wide varitey of tissues, are a possible pathway of citrulline formation in skeletal muscle. Other possible sources could be citrulline released from muscle protein degradation (formed by peptidylarginine deiminase) or citrulline synthesis by arginine deiminases. Constraints inherent in methods for measuring arginine, citrulline, urea and total protein degradation could limit sensitivity or possibly prevent analysis in experiments designed to detect arginine metabolism in skeletal muscle.

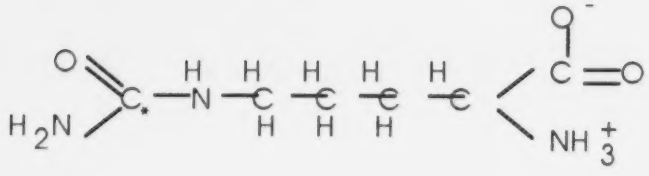
The suggestion that enzymes producing citrulline may be present in normal skeletal muscle raises the question of their physiological function. Nitric oxide in other tissues is a regulator, if produced in muscle, what does it regulate? Similarily, what would be the purpose of any other enzymes which synthesize citrulline? Does citrulline have a role in skeletal muscle? If these enzymes are active, then muscle requirements for arginine and implications of those requirements for whole body arginine flux and total arginine requirement are of interest. The eventual fate of citrulline is also of interest.

Figure 1.1: Structures of Arginine and Citrulline

The asterisk marks the guanido carbon of arginine and the ureido carbon of citrulline. Drawings are adapted from reference 23.



ARGININE



CITRULLINE

Figure 1.2: The Nitric Oxide Synthase Metabolism of Arginine

Nitric oxide synthase metabolizes arginine into citrulline and nitric oxide. It has been determined that oxygen is derived from molecular oxygen, not water, and that the first intermediate is N-hydroxyl-arginine. The subsequent steps which result in citrulline and nitric oxide have not been clearly resolved. Diagram is adapted from reference 23.

Figure 1.3: The Urea Cycle

Diagram adapted from references 2 and 29.

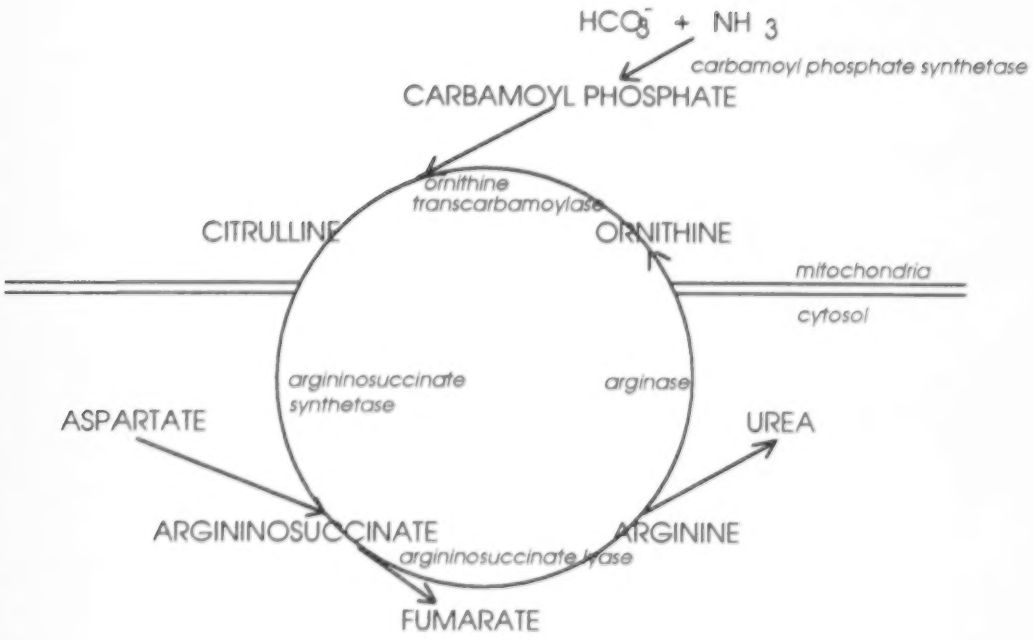
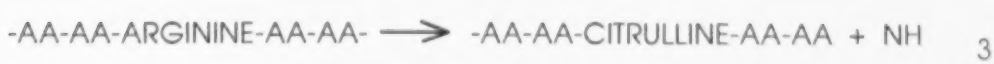


Figure 1.4: Arginine Deiminase Metabolism of Arginine

The arginine deiminases metabolize arginine to citrulline with the release of ammonia.

a) Peptidylarginine Deiminase

The substrate is protein bound arginine. Diagram adapted from reference 39.



b) Arginine Deimininase

The substrate is free arginine. Diagram adapted from reference 43.

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II. RESEARCH PLAN

RATIONALE

Skeletal muscle is a large organ and therefore metabolism in this tissue has possible implications for the entire body. No systematic study of arginine metabolism or citrulline synthesis in intact skeletal muscle has been made. If arginine is metabolized to nitric oxide in muscle, discovering its physiological role would lead to a greater understanding of how skeletal muscle function is regulated. A relevant first step is to confirm the presence of nitric oxide synthase in muscle.

HYPOTHESIS

It is hypothesized that nitric oxide synthase is active in skeletal muscle. Specific hypotheses are:

1. Citrulline is synthesized in skeletal muscle.
2. Arginine is the metabolic source of citrulline in skeletal muscle.
3. Nitric oxide synthase is the enzyme responsible for the metabolism of arginine to citrulline in skeletal muscle.

OBJECTIVES

The objectives of this thesis are:

1. To measure citrulline release from muscle in the altered physiological states of exposure to endotoxin or trauma *in vivo*.
2. To measure citrulline release from muscle in altered pharmacological states *in vitro*, ie: presence or absence of calcium, the potential enzyme substrates arginine and ornithine and a protein synthesis inhibitor.
3. To measure loss of citrulline from the intracellular pool during muscle incubation.
4. To measure protein-bound citrulline in skeletal muscle.
5. To measure radiolabelled citrulline and urea synthesis using [*guanido*-^{14}C]arginine as a substrate in intact muscle and in muscle cytosol.
6. To measure radiolabelled citrulline synthesis in the presence of a nitric oxide synthase inhibitor in intact and in muscle cytosol.

To achieve these objectives several methodological questions had to be answered. The specific objectives are:

7. To determine if prelabelling of proteins provides a direct measure of protein degradation.
8. To shorten the time required to analyse samples for citrulline content using HPLC.
9. To measure loss of citrulline during acid hydrolysis of proteins.
10. To separate citrulline from arginine and urea using cation exchange chromatography.

THESIS ORGANIZATION

Chapter I gives a brief overview of the current knowledge of amino acid metabolism in skeletal muscle, including protein synthesis and degradation. It also describes all currently known metabolic pathways of citrulline synthesis.

This chapter lists the specific hypothesis and objectives and describes the layout of the thesis.

In Chapter III several experiments measuring protein degradation using *in vivo* prelabelling of proteins are described (Objective 7).

Methods for measuring citrulline (Objectives 8-10) are described in the fourth chapter.

The fifth chapter describes a series of experiments to measure citrulline and urea release or synthesis in whole muscle incubations or in muscle cytosol (Objectives 1-6).

In the final chapter (VI), the conclusions of the previous chapters are summarized, areas for future research are discussed, and speculations are made on the possible role of NOS in skeletal muscle.

The first appendix gives detailed procedures for preparing reagents and samples used in the methods described in Chapter IV.

The second appendix gives the amount of citrulline synthesized in the presence of various concentrations of L-NAME or L-NMMA. The data in the table is calculated from released radioactivity which is presented in Fig. 5.2.

III. DETERMINATION OF MUSCLE PROTEIN CATABOLISM

INTRODUCTION

This chapter presents the results of experiments designed to optimize the study of muscle protein degradation. The technique presented here would be useful in studies to determine if nitric oxide or other factors influence the rate of muscle protein catabolism. Protein turnover (or balance) is the sum of protein synthesis and protein degradation. Both muscle protein degradation and synthesis are closely and independently regulated, so that understanding muscle protein balance depends on study of both these processes. Protein synthesis has been studied *in vitro* or *in vivo* with very little technical or conceptual difficulty (reviewed in 1). The main difficulty in studying protein degradation is that its products (i.e.: free amino acids) are rapidly removed either for protein synthesis or amino acid catabolism. Described here are some considerations and experimental studies of muscle protein degradation.

Three main approaches have been used for study of protein catabolism:

A.) Calculate total protein degradation from simultaneous measurements of protein synthesis and net protein balance (2). Net protein balance (positive or negative) = synthesis - degradation. In this approach, protein synthesis is determined as incorporation of labelled amino acids into protein. Protein balance is measured as the net release (uptake) of amino acids and degradation is calculated by the difference. Calculations of degradation in this approach incorporate errors inherent in both component measurements, decreasing sensitivity.

B.) Block protein synthesis and measure total degradation as the net release of amino acids. An alternative approach to measuring protein degradation used in cells *in vitro* is to simply block reincorporation of amino acids by the use of protein synthesis inhibitors such as cycloheximide (1). Overall rates of muscle protein degradation determined this way agree reasonably well with data obtained with the approach described above (2). However, the use of cycloheximide would normally lead to rapid depletion of tissue proteins with high turnover rates, leading to questions about viability and the ability of the tissue to express normally regulated protein degradation. For example, Fagan and Goldberg (3) demonstrated that the synthesis of prostaglandins in response to various stimuli is mediated by a protein with a very short half-life, and that in skeletal muscles incubated in the presence of cycloheximide, prostaglandin synthesis is almost completely inhibited. Prostaglandins are mediators of activated muscle protein catabolism, but this cannot be studied in the presence of inhibitors of protein synthesis. Similarly other treatment effects on protein degradation mediated by short-lived proteins would be prevented by blocking protein synthesis.

C.) Amino acid release from radiolabelled protein. In cells in tissue culture, a third approach was developed where cell protein was labelled by a pulse of radioactive precursor. After a defined labelling period, the precursor was withdrawn and unincorporated labelled precursor washed out. Following this wash, the rate of release of radioactivity is measured in the presence of a high concentration of unlabelled precursor. The relative amounts of unlabelled precursor compared to labelled amino acid released from protein degradation ($\sim 10^6$:1) is so great as to abolish reincorporation of the labelled amino acid. Garlick *et al.* (4) list the three assumptions underlying this method: 1) that steady state is maintained throughout the experiment, 2) that turnover rates of the protein remain constant throughout the experiment, and 3) that there is no recycling of radiolabelled amino acid in protein synthesis.

This method was used earlier in cells in tissue culture (5) and has been used for *in vitro* skeletal muscle. Wu and Thompson (6) injected radiolabelled phenylalanine into chicks 24 hr prior to harvesting muscles for *in vitro* muscle incubation experiments. Beck *et al.* (7) used the same method to measure protein degradation in tumor bearing mice. By injecting radiolabelled phenylalanine, physiologically labelled muscle proteins were generated. Isolated tissues, subsequent to washing and incubation in the presence of a high concentration of unlabelled phenylalanine, would release label from protein at a rate related to the degradative rate. The release of radioactivity from a tissue containing labelled proteins would be characteristic of the range of half lives of those proteins. Rapidly turning over proteins would lose label quickly and after several half lives have elapsed no further label will come from this source. By contrast, long lived proteins would continue to release label. Thus early in

incubation the release of radioactivity would be greater than later, mainly from rapidly turning over proteins.

The prelabelling method has the potential to be a direct, sensitive measure of total protein degradation but it needs to be verified in rat skeletal muscle. The first two of Garlick's assumptions have been tested and found valid in the *in vitro* muscle incubation systems (8-11). The following experiments test the assumption that there is no recycling of label, measure the repeatability of the method, and assess the response of muscle protein degradation measured in this way to free calcium, a factor with established effects on muscle protein degradation.

MATERIALS AND METHODS

Chemicals

Alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, glucose, cycloheximide, HEPES, and insulin (bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TCA was purchased from Anachemia (Vancouver, B.C., Canada). Diethyl ether was purchased from BDH (Toronto, ON, Canada). L-[2,6-^{3}H]phenylalanine was purchased from Amersham (Oakville, ON, Canada). Ecolite™ was purchased from ICN, (Costa Mesa, CA, U.S.A.). Hionic-flour™ and Soluene-350™ were purchased from Packard (Meriden, CT, U.S.A.).

Animals

Experiments were carried out in compliance with the guidelines of the Canadian Council of Animal Care. Male or female Sprague-Dawley rats (approximately 65 g body weight) of the Buffalo strain were obtained from colony maintained in the Department of Animal Science, University of Alberta. Rats were group housed in plastic bottomed cages and offered laboratory chow and water *ad libitum*. Rat rooms were held at constant humidity (80%) and temperature (24^{o}C).

Muscle Incubation

Either quarter diaphragms (10), epitrochlearis (11), soleus (12) or extensor digitorum longus (12) muscles were used in the incubations. After rats were killed by CO_2 asphyxiation, muscles were rapidly dissected, weighed, and placed in 3 ml incubation media in a shaking water bath (36^{o}C). After a pre-incubation (30 min, unless otherwise noted) muscles were transferred to and incubated in 3 ml fresh media. Incubation times are noted with the results of each experiment. Unless otherwise noted preincubation media was the same as incubation media: supplemented Krebs-Ringer bicarbonate medium (119 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.25 mM NaH_2PO_2, 2.0 mM HEPES, plasma levels of amino acids (13), 5.0 mM glucose and 0.5 U/ml insulin), oxygenated continously with a mixture of O_2 and CO_2 (19:1). Calcium free medium included 1 mM EGTA and excluded $CaCl_2$. Cycloheximide was added at 0.5 mM to media of one experiment. At the end of incubation, the muscle was placed in 1 ml of ice-cold 2% TCA, and the muscle and the media were frozen (-20^{o}C). Muscles were homogenized with a glass/glass homogenizer, rinsed with 1 ml of 2% TCA. The homogenate was allowed to stand at 4^{o}C for about 1 hr, then centrifuged at 1000 x g for 10 min. The TCA soluble fraction containing free amino acids in the intracellular and extracellular fluids was stored frozen until analyzed. When the TCA insoluble protein fraction was analyzed, it was washed twice with 1 ml of 2% TCA and then with diethyl ether, and allowed to dry.

Protein Synthesis Measures

Protein synthesis was measured similarly to Fulks *et al.* (8). Briefly, muscles were incubated in supplemented KRB containing 1 mM phenylalanine, to which was added L-[2,6-^{3}H]phenylalanine (24.3 μCi/ nmol phenylalanine). At incubation end (2 hr), the specific activity of the TCA soluble fraction was measured as the precursor pool for protein synthesis. Protein bound radioactivity was counted in muscle homogenates that had been solubilized in 0.75 ml Soluene. Six ml of a chemiluminescence quencher (Hionic-flour™) and 8 ml of a scintillation fluor (Ecolite™) were added to solubilized muscles and radioactivity was counted in a Packard 1600CA Tricard Liquid Scintillation

analyzer. Protein synthesis was calculated by dividing protein bound radioactivity (dpm) by TCA soluble specific activity (dpm/nmole phe).

Measuring Protein Degradation

Rats were injected with 0.065 - 0.175 mCi [^{3}H]phenylalanine in 6.0 mM phenylalanine in sterile saline. The injection was given intraperitoneally once per day for 3 days. Muscle contains a large proportion of slowly turning over myofibrillar protein and multiple injections were used to increase the specific radioactivity of the slowly turning over proteins. Rats were killed on the fourth day and the muscles were rapidly dissected. Muscles were washed at least twice in supplemented KRB to remove any free label from the intracellular and extracellular fluid. Radioactivity in the media and TCA soluble muscle fraction was counted after adding Ecolite™ and summed as the amount of protein bound label released. The TCA insoluble fraction was hydrolyzed and aliquots were counted for radioactivity. Protein degradation data were expressed as %/2 hr and calculated as follows:

$$\%/2\ hr = \frac{\text{dpm released /2 h x 100}}{\text{total dpm}}.$$

Absolute values for total protein degradation (nmol phe/2 hr) were calculated by dividing dpm released by the specific activity of protein bound phenylalanine (dpm/nmol phe).

High Performance Liquid Chromatography (HPLC)

Phenylalanine was measured in TCA soluble tissue fraction and muscle protein by HPLC. The procedure was modified from Jones and Gilligan (14). Samples were mixed with the fluoraldehyde (o–phthaldialdehyde) reagent immediately before injection. Twenty-five µl of sample and reagent were injected onto a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with Supelco LC-18 reverse phase packing (20-40 µm). A Varian 5000 HPLC and Varian Fluorichrom detector (excitation 340 nm, emission 450 nm) were used to separate and measure the amino acids. Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom Chromatography data system. The two solvents used were 0.1 M sodium acetate and methanol. β-amino-butyric acid was used as an internal standard. Gradient flow was 1.5 ml/min and began with 38% methanol increasing to 50% methanol at 6 min. From 6.1 to 6.9 min the gradient used 80% methanol, going to 38% methanol at 7 min. Total run time was 12 min. Peaks were identified and quantified by running standards of known amino acids and blanks of appropriate incubation media with each group of samples.

Statistical Analysis

Statistical analyses used were a multiway ANOVA, using rat as a source in the model and variation due to rat was removed where appropriate. Repeated measures analysis was used to analyze time course experiments. If three means were significantly different as determined by ANOVA, means were separated by a Least Significant Differences test. If $p<0.05$, results were considered significantly different. (SAS 6.06.01, SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

The assumption that radiolabelled phenylalanine is not recycled into newly synthesized proteins was proven to be valid since in washed, incubated muscles fractional degradation rates were identical in the presence and absence of cycloheximide, an inhibitor of protein synthesis (4.75 vs. 5.02%/2 hr, S.E.M.=0.09, $p=0.1047$, $n=8$). It is also important to ensure that free label is washed out of the extracellular free pool. This was demonstrated by washing quarter diaphragms either for six 5 min periods or for two 15 min periods (Table 3.1). Total radioactivity released into the media was not significantly different between the two procedures and as seen from the data, radioactivity release has leveled off after the first wash. As well, the washing method had no effect on radioactivity release into

the incubation media or on tissue free radioactivity during the subsequent incubation period (data not shown). From this it was concluded that two 15 min washes were adequate.

Three experiments were performed to assess the rate of protein degradation. In all three, the fractional degradation rate was significantly higher in the first period than in subsequent periods (Table 3.2). In the subsequent two periods, there was no significant difference in fractional degradation rates. It is unlikely that release of radioactivity from the incubated muscles declined over time because the tissue was becoming unviable, since in a parallel experiment protein synthesis was linear over the three periods (data not shown).

Another study was conducted to determine whether the tissues responded to a well-characterized modifier of proteolysis. When soleus muscle pairs were incubated with 0 or 3 mM calcium, addition of calcium increased fractional degradation by 58% (4.44 vs. 7.59%/2 hr, S.E.M.=0.10, p=0.0001, n=8). This difference is similar to that (77%) observed by Rodemann *et al.* (15) who measured total protein degradation in diaphragms.

DISCUSSION AND CONCLUSION

An assumption that remains to be tested is that the released radioactivity is associated with phenylalanine. Phenylalanine can be degraded to tyrosine and eventually to acetyl CoA. To use radioactivity as a measure of protein degradation it must be determined that the released radiolabel is primarily associated with phenylalanine released from degraded protein and not with its possible metabolites.

Others who have measured total protein degradation by tyrosine release in the presence of cycloheximide or calculated it after measuring synthesis and net degradation, have demonstrated that total protein degradation is linear with time. Fulks *et al.* (8) measured net tyrosine and total tyrosine release in the presence of cycloheximide from quarter diaphragms for 3 hr and both were linear. Rodemann and Goldberg (16) had similar results over 2 hr while Hasselgren *et al.* (17) showed that extensor digitorum longus and soleus muscles from the rat released tyrosine linearly over 2 hr in the presence of cycloheximide. In the chick extensor digitorum communis muscle net protein degradation and protein synthesis were linear for 9 hr (9) therefore total degradation must also be linear. The results reported here are not linear, probably because the high rates of fractional degradation early in the incubation reflect the rapid turnover of the most short-lived proteins, which rapidly go to a low specific radioactivity because their resynthesis does not include [^{3}H]phenylalanine. It would be anticipated that the lower, but more constant release of radioactivity in later incubation periods reflects the degradation of proteins with longer half lives. The fall in released radioactivity was also observed by Millward (18) in gastrocnemius muscles *in vivo* over 7 days.

Previous measurements of total protein degradation in skeletal muscle using cycloheximide range from 0.28-0.86 nmol phe/mg tissue/2 hr (8,16,20) (converted to nmole phe by multiplying nmol tyr by 1.408 (2)). Total protein degradation calculated from simultaneous measures of protein synthesis and net protein balance ranges from 0.49-1.12 nmol phe/mg tissue/2 hr (2,21). The values calculated using prelabelled proteins (1.75 ± 0.09, 0.83 ± 0.09, 0.90 ± 0.09 nmol phe/mg tissue/2 hr for periods 1, 2 and 3 respectively in the experiment in which 0.125 mC/rat/day were injected) are at the higher end of the range of previous measures, reflecting the rapid degradation of short-lived protiens.

These results verify that prelabelling proteins is a sensitive method for measuring total protein degradation without blocking protein synthesis. This method is not affected by changes in protein synthesis, and is affected by changing the calcium availability, as expected due to the activity of calcium activated proteases. Non-linearity would not interfere with the interpretation of the results if an experimenter wished to examine relative effects of treatments added to the incubation media. This method may also provide the opportunity to study short and long lived proteins by using a double label technique or by examining the two areas of the curve as done *in vivo* by Arias *et al.* (19). The major advantage of this method is that it provides a sensitive, direct measure of protein degradation while avoiding use of potentially toxic protein synthesis inhibitors.

TABLE 3.1: Washing of Incubated Muscles to Remove Free Radiolabel

Rats were injected with 0.125 mCi L-[2,6-^{3}H]phenylalanine/rat on each of three days. On the fourth day quarter diaphragms were dissected and incubated in supplemented KRB (see text) for six periods of 5 min or for 2 periods of 15 min. Radioactivity in the incubation media was counted, and cumulative counts were statistically analyzed (multiway ANOVA). Data is presented as means, n=4, pooled S.E.M. were computed using cumulative means at 15 and 30 min. Different superscripts within rows and columns indicate significant differences, $p \leq 0.05$.

	Cumulative Counts Released to Washing Media (dpm/mg tissue)		
Wash Time (min)	Two Washes	Six Washes	Pooled S.E.M.
5		72.6	
10		84.1	
15	79.2[a]	93.4[a]	8.2
20		101.8	
25		108.3	
30	99.2[b]	115.4[b]	8.2

TABLE 3.2: Fractional Degradation of Protein from Incubated Skeletal Muscles

Fractional degradation was measured as the percentage of total radioactivity released from incubated quarter diaphragms. Muscles had been previously radiolabelled by intraperitoneal injection of tritiated phenylalanine for the three days prior to incubation. Data are presented as means ± S.E.M., columns with different superscripts are significantly different, $p \leq 0.05$. ND=not determined

Injection Dose (mCi/rat/day)	Radioactivity (dpm/mg/period one)		n	Wash Time (min)	Period Length (min)	Fractional Degradation Rate (%/period)			
	Protein- Bound	Released to Media				Period			
						One	Two	Three	S.E.M. (pooled)
0.175	5111 ± 243	175 ± 10	5	2 X 15	120	5.27^{a}	3.62^{b}	ND	0.17
0.125	6445 ± 649	269 ± 27	8	2 X 15 or 6 X 5	120	3.86^{a}	2.48^{b}	2.19^{b}	0.23
0.065	1834 ± 221	80 ± 11	8	1 X 15 and 1 X 60	90	4.38^{a}	2.32^{b}	2.06^{b}	0.20

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IV. METHODS FOR MEASURING CITRULLINE

INTRODUCTION

Nitric oxide is active in maintaining vasodilatory tone, neural activity and when released in larger amounts, cytotoxicity (1). Nitric oxide is synthesized from arginine, with citrulline as a by-product, through the activity of nitric oxide synthase (reviewed in 1-4). Measuring nitric oxide is therefore a direct measure of NOS activity. Because of its short half life (~5 sec)(3) measuring nitric oxide directly is difficult. A method (5), based on the Griess reaction which detects nitrite, has been developed to measure nitrite and nitrate, more stable metabolites of nitric oxide (2). In the muscle incubation system used for most of the experiments presented in this thesis, released citrulline concentrations range from 0.5 to 3.0 μM (Chapter 5). Assuming that the ratio of citrulline to nitrate plus nitrite is 1:1, total nitrite and nitrate would still be at the lower limits of detection of the modified Griess reaction (range: 1 - 300 μM)(5). As an alternative, it was decided to measure citrulline as a measure of NOS activity. Citrulline can be separated from other amino acids and measured using the HPLC method of Jones and Gilligan (6). This method was shortened to end after the amino acids of interest (citrulline, arginine and occasionally tyrosine) were eluted.

Several other enzymes can synthesize citrulline (reviewed in Chapter 1). To measure the amount of citrulline contributed by peptidylarginine deiminase, it was necessary to measure the amount of citrulline in skeletal muscle protein. Before using the modified HPLC method to measure citrulline in protein, acid hydrolysis was used to degrade the protein into the component amino acids. Citrulline is hydrolysed into ornithine during this process (7), and so a sequential hydrolysis was performed in order to determine the amount of citrulline lost during the acid hydrolysis procedure. To eliminate urea cycle enzymes as a source of citrulline, [*guanido*-^{14}C]arginine was used as a substrate (see Fig. 5.1). To measure citrulline radioactivity, it must be separated from the substrate, arginine, and other potential radioactive metabolites of arginine. The ion exchange chromatography method of Gopalakrishna and Nagajaran (8) was developed to separate arginine from its metabolites: ornithine, citrulline, urea, polyamines, glutamic acid, proline and glutamine in an acid media. The muscle incubation media is a physiological buffer (pH 7.4) containing dissolved electrolytes and so the published method was modified to ensure separation of arginine, citrulline and urea dissolved in incubation media. This chapter presents the modified methods for HPLC measurement of citrulline, sequential acid hydrolysis and cation exchange chromatography.

MATERIALS AND METHODS

Chemicals

Brij 35™, trisodium citrate, sodium hydroxide, hydrogen chloride, potassium hydroxide, acetic acid, tetrahydrofuran, HPLC grade water and HPLC grade methanol were purchased from BDH (Toronto, ON, Canada). Trichloroacetic acid, benzoic acid and sodium acetate were purchased from Fischer Scientific (Fairlawn, N.J., U.S.A.). L-[*guanido*-^{14}C]arginine and L-[*ureido*-^{14}C]citrulline were purchased from Dupont New England Nuclear, (Mississauga, ON, Canada). [^{14}C]urea was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.). Ion exchange resins AG1-X8 (200-400 mesh, acetate form) and AG50W-X8 (200-400 mesh, H^+ form, analytical grade) were purchased from BioRad Laboratories (Richmond, CA, U.S.A.). Scintillation cocktail, Ecolite™, was purchased from ICN (Costa Mesa, CA U.S.A.). All other supplies were purchased from Sigma Chemical Co. (St. Louis, MO U.S.A.).

HPLC Separation of Citrulline

Jones and Gilligan (6) developed a method to measure amino acids using reverse phase HPLC. Amino acids are derivatized with a fluoraldehyde compound that will fluoresce under certain wavelengths, and applied to a column packed with silica-based packing. A standard procedure, based on this method, has been established in the Animal Science Lab, University of Alberta. Citrulline elutes at

~25 min, approximately halfway through the run, and this method was modified to end shortly after citrulline was eluted, shortening the total run time.

Sample and Standard Preparation

Citrulline dissolved in incubation media, TCA soluble muscle homogenates, 6 M HCl, or in 0.5 M sodium citrate can be measured. Amino acid standards are prepared in solvents as close as possible to the samples. This avoids amino acids running at slightly different times in standards compared to samples and avoids having to calculate adjustments for dilution differences. Aliquots of sample and standards were buffered to pH 9-10, but not greater. Detailed directions for reagent, sample and standard preparation are given in Appendix 1.

Sample Analysis

Buffered sample aliquots were mixed 1:1 with flouraldehyde reagent (1.0 g o-phthaldialdehyde, 25 ml methanol, 225 ml 0.4 M sodium borate, 1 ml 2-mercaptoethanol and 8 ml Brij 35™) before injection. The mixed sample and fluoraldehyde reagent (25 µl) was injected onto a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with Supelco LC-18 reverse phase packing (20-40 µM). A Varian 5000 HPLC and Varian Fluorichrom detector (excitation 340 nm, emission 450 nm) were used to separate and detect the amino acids. Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom Chromatography data system. The solvents were used in the gradient as listed below, with a flow rate of 1.5 ml/min. Total run time per sample was 34 min.

Gradient:

Time (min)	% Solvent A (0.1 M sodium acetate)	% Solvent B (100% methanol)
0.0	100	0
0.1	86	14
22.0	85	15
22.1	70	30
26.0	69	31
26.1	0	100
28.0	0	100
28.1	100	0

The amount of tyrosine, an amino acid that is neither synthesized nor metabolized in skeletal muscle, was compared to the amount of citrulline in incubation media and skeletal muscle protein. From these ratios, the amount of citrulline released by muscle protein degradation was calculated (see Chapter 5, page 34). To measure tyrosine it was necessary to clearly separate tyrosine from the other amino acids that elute after citrulline. An alternate gradient was developed to be able to measure both citrulline and tyrosine with a total run time of 39 min. Alanine elutes before tyrosine so it, too, is separated.

Gradient:

Time (min)	% Solvent A (0.1 M sodium acetate)	% Solvent B (100% methanol)
0.0	100	0
0.1	86	14
22.0	85	15
22.1	70	30
26.0	69	31
26.1	53	47
31.4	51	49
31.5	0	100
33.5	0	100
33.6	100	0

Sequential Hydrolysis of Citrulline

Proteins can be degraded into their component amino acids by using acids and moderate temperatures. This process may alter some of the original amino acids (9). In order to define how much tyrosine or citrulline was lost during protein hydrolysis, standards containing tyrosine and citrulline standards were subjected to acid hydrolysis for varying times from 0 to 30 hours. Steinert *et al.* (7) measured citrulline loss during hydrolysis using proteins containing citrulline or a comparable mixture of free amino acids with identical results in both cases, suggesting that rates of hydrolysis do not vary between free citrulline and citrulline in peptide bonds.

A standard containing 2.02 x 10^{-7} mol tyrosine and 1.98 x 10^{-7} mol citrulline/ml was prepared. Duplicate vials for each time point were prepared by placing 1 ml of standard in a glass screw-top vial to which 3 ml of 6 M HCl was added. The vial was shaken, sparged with N_2, and capped. Duplicate vials for time zero were held at 4^{o}C, and the remaining vials were placed in a 110^{o}C oven. After 1 hr all caps were checked and tightened if necessary. Duplicate vials were removed at approximately 3, 6, 9, 12, 18, 24 and 30 hr, and held at 4^{o}C until HPLC analysis. Measured amounts of citrulline and tyrosine were plotted against actual time (min) in the oven. Results were analyzed using a linear regression program (SAS 6.06.01, SAS Institute Inc., Cary, NC, U.S.A.).

Cation Exchange Separation of Arginine and its Metabolites

Cation exchange chromatography separates molecules on the basis of charge. Charged molecules, including amino acids, are attracted to a column of opposite charge, and then are eluted in a solvent whose components have a higher affinity to the column than the molecule of interest. The published cation exchange chromatography method (8) separates arginine from all its potential metabolites (9). If [*guanido*-^{14}C]arginine is used as a substrate, label can be found in urea and citrulline, but not in ornithine (Fig 5.1) or its subsequent metabolites: glutamine, glutamate, proline or polyamines (9). Therefore, only arginine, citrulline and urea need to be separated from each other. Most of the samples to be tested were in physiological buffer (pH 7.4) containing electrolytes, glucose, and insulin (Chapter 5, page 32), but the published method was used on acidic samples. A simplified procedure was prepared and tested to ensure that amino acids and urea dissolved in incubation media could be separated.

Preparation of Samples, Reagents and Columns

Two types of samples were separated by cation exchange. Samples in incubation media were applied to the columns without any further preparation. Samples in TCA were adjusted to pH 6 with 5 N KOH. Detailed procedures for preparing the reagents are given in appendix 1.

Samples were separated on a sequence of two columns (Fig. 4.1). Each column was made in the syringe sleeve of a plastic 3 ml syringe (internal diameter 0.8 cm). Loosely packed glass wool was put in the bottom 0.2-0.4 ml of the syringe. Cation exchange resin (AG50W-X8, 200-400 mesh, H^+ form, analytical grade) was prepared in two ways. For the upper column the resin was suspended in 5 volumes of 2 M NaOH (Na^+ form), and for the lower column, resin was suspended in 2 M HCl (H^+ form). The

resin was allowed to settle, and the supernatant slowly poured off. Resin was rinsed several times with water. Suspended resin was pipetted into the columns above the glass wool until the resin bed occupied 1-1.1 ml. Column packing took place under gravitational force. Water was pipetted onto the top of the column until the eluent draining off the column was pH 6-7 (same as applied water). Columns were then mounted with the Na^+ column above the H^+ column. Columns were regenerated (see below) and reused 5 times before being discarded.

Method

The sample was applied to the top of the Na^+ column (Fig. 4.1) with a pipette, and allowed to percolate through the Na^+ column onto the H^+ column by gravitational force. Five ml of water was then applied to the top of the Na^+ column and allowed to drip through both columns. Eluent was discarded. Columns were then separated.

Na^+ Column: Five ml of 0.15 M trisodium citrate (pH 6) were applied and discarded. This fraction contained ornithine. Arginine was collected by applying 10 ml of 0.5 M trisodium citrate (pH 6.5), discarding the first 2 ml. The column was cleaned and regenerated by applying 4 ml of 2 M NaOH and then rinsed with water until the eluent was pH 6-7.

H^+ Column: Urea was eluted by applying 18 ml of water. The first 6 ml and the last 4 ml were discarded. Citrulline was eluted with 3 ml of 6 M HCl. The column was cleaned and regenerated by applying 2 ml of 2 M HCl, then rinsing with water until the eluent was pH 6-7.

Aliquots and Analysis

Aliquots were collected in 18 ml plastic scintillation vials. From the arginine and urea fractions 3 ml was removed to bring the sample volume in the scintillation vial below 30% total volume as suggested in the manual for the scintillation fluor (Ecolite™). The 3 ml portion was stored at -20°C in disposable borosilicate tubes until HPLC analysis. Only 1 ml was removed from the citrulline fraction. To the sample remaining in the scintillation vials, 11 ml of Ecolite™ was added. After the lids were put on securely (label lids, not vials, to avoid label interfering with the light path in the counter), the vials were turned over several times to thoroughly mix samples. Vials are too full for vortexing to adequately mix the samples. Sample counting in the liquid scintillation counter (Packard 1600CA TriCard Liquid Scintillation Analyzer) was terminated at 2% uncertainty at a 95% confidence interval or at a maximum of 20 min. Counting efficiency was ≥ 90%.

Before columns were used to separate samples, recoveries were tested by adding radiolabelled amino acids to incubation media containing 1.0 mM arginine, 0.06 mM citrulline and 0.08 mM urea. [^{14}C]arginine was added at 0.18 μCi/ml medium, while [^{14}C]citrulline or [^{14}C]urea were added at 0.011 μCi/ml medium.

RESULTS AND DISCUSSION

The modified HPLC method clearly acheives baseline separation of the amino acids aspartate, glutamate, serine, histidine, glutamine, glycine, threonine, citrulline and arginine (Fig. 4.2). The remaining amino acids are eluted in two large peaks at the end of the run. In addition, alanine and tyrosine are clearly separated if the alternate gradient (page 23) is used (not shown). These modifications save time (32 and 22% respectively) compared to the original method, allowing more analyses per day, as well as saving solvent and subsequent waste. This method was used for all HPLC measurements of amino acids reported in Chapter 5.

Acid hydrolysis caused the loss of citrulline with time (Fig. 4.3) (r^2=0.83, $p<0.0001$). Using the regression formula calculated from the regression analysis, it was determined that 47% of the citrulline was lost after 24 hr. This result is similar to that reported by Steinert *et al.* (7)(~40%). There was no significant loss of tyrosine over 30 hr ($p=0.0954$, r^2=18.5%). The minimum citrulline detectable by HPLC (39 pmol/ml sample) was divided by 47%, and the amount (~44 mg) of protein hydrolysed to calculate minimum citrulline detectable per mg muscle (38 pmol/mg). This figure was used in Chapter 5 to determine tyrosine:citrulline ratios in muscle protein and compare them to the ratio in incubation media.

Cation exchange chromatography was successful in separating arginine, citrulline and urea from each other (Table 4.1). Only a small proportion of the arginine co-eluted in the citrulline or urea fractions (Table 4.1), but since arginine specific activity was high, the radioactivity due to arginine could approach that due to either citrulline or urea. Therefore, for each experiment, backgrounds were established by running incubation media blanks (media that had been incubated without a muscle) through the columns. The average background was subtracted from the sample value to give actual citrulline or urea radioactivity. The contamination of citrulline by urea and vice versa is minimal. Though part of the urea and arginine fractions were discarded the bulk of label is collected. No correction factors were used to correct for either partial recovery or for contamination by citrulline or urea.

The modified method uses the same eluants as the original method (8), only the volumes have been increased. Therefore the modified method was used to separate arginine, citrulline and urea in the TCA soluble portion of muscle homogenate. Run time, from sample application to the end of column regeneration and rinsing was approximately 3.5 hr. It was manageable to run ten columns at a time.

The modified cation exchange method is simpler than the original since one less column is used and three fewer fractions are collected. It has been demonstrated that the modified method effectively separates arginine, citrulline and urea dissolved in a physiological buffer, therefore it is an appropriate method for measuring radiolabelled citrulline in the muscle incubation system. In addition, the amount of citrulline lost during acid hydrolysis was measured, and the HPLC method for measuring citrulline was successfully shortened.

Figure 4.1: Schematic Diagram of Cation Exchange Apparatus and Procedure

The cation exchange separation of arginine and its metabolites was modified (see text) from Reference 8.

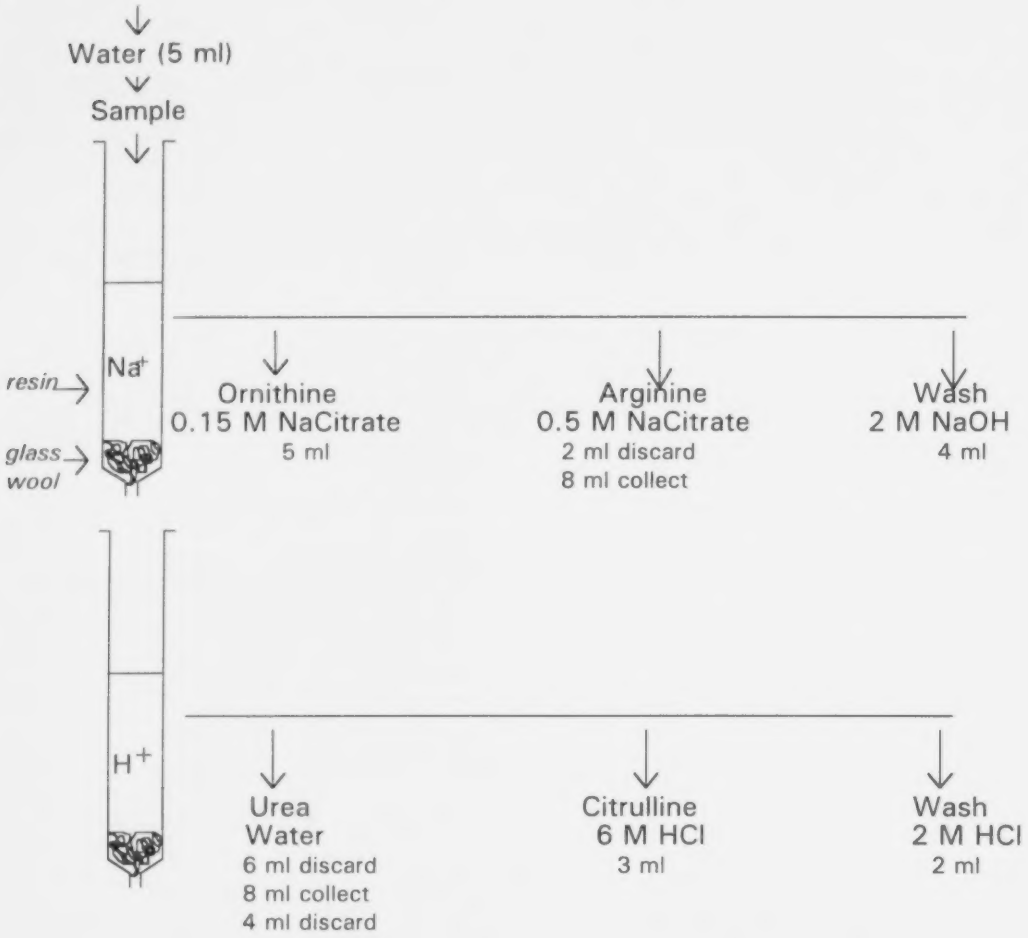

Figure 4.2: HPLC Separation of Amino Acids Including Citrulline

This chromatograph shows the HPLC separation (detailed in text) of an amino acid standard. AGPA refers to the internal standard, L-α-amino β-guanidino proprionic acid.

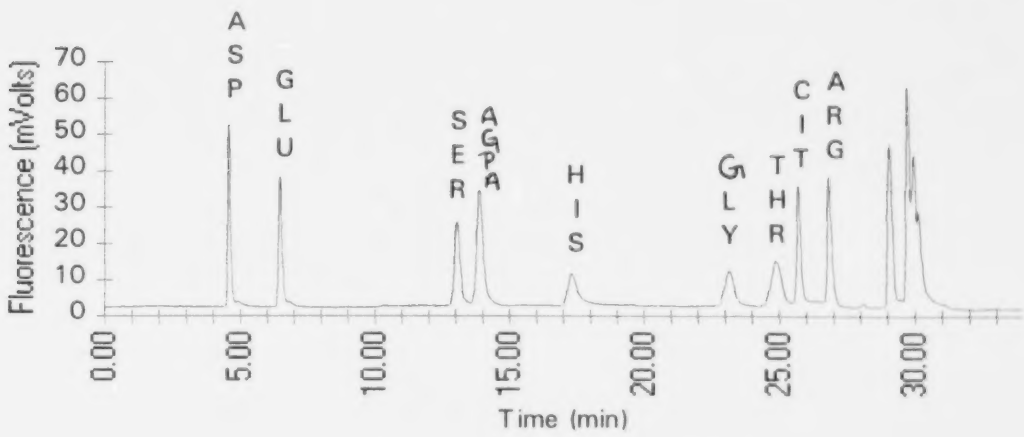

Figure 4.3: Sequential Loss of Citrulline During Acid Hydrolysis

Standards containing free citrulline and tyrosine were subjected to acid hydrolysis for 0 to 30 hours as stated in the text. Regression equation is: $y = 105 - 0.0347x$, $r^2 = 83\%$, $p < 0.0001$. The solid line shows the citrulline loss predicted by the regression equation.

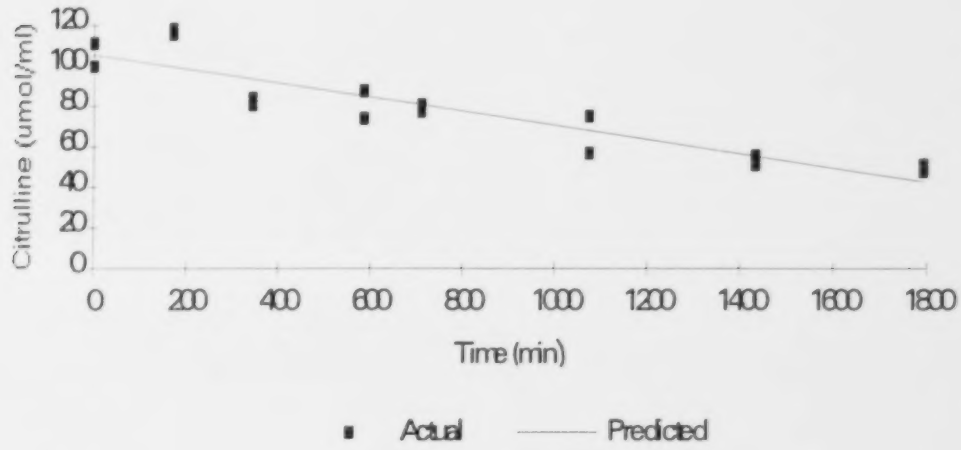

Table 4.1: Modified Cation Exchange Separation of Arginine, Urea and Citrulline, Using Radioisotopically Labelled Compounds.

To test product recoveries using the cation exchange method, radioisotopically labelled arginine (0.18 μCi/ml), citrulline and urea (0.011 μCi/ml) were added to supplemented KRB containing 5.0 mM glucose, 0.5 U/ml, 1.0 mM. arginine, 0.06 mM citrulline, and 0.08 mM urea. Samples were separated using a sequence of two cation exchange columns as described in the text. The data presented (n=1) are from the final method which was refined using several separations, all with similar values.

	Recovery of Label in each Fraction (% of label applied)			
Radioisotope added to sample	Fraction	Arginine	Citrulline	Urea
Arginine		76.9	1.3	0.4
Citrulline		0.2	97.2	1.4
Urea		2.1	0.9	88.8

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V. METABOLISM OF ARGININE AND SYNTHESIS OF CITRULLINE IN RAT SKELETAL MUSCLE

INTRODUCTION

Arginine and citrulline are two closely related amino acids. Citrulline is both a major metabolite of arginine, and its biosynthetic precursor. Arginine and citrulline are easily interconverted by the hepatic enzymes of the urea cycle (reviewed in 1). The *de novo* biosynthesis of arginine requires net citrulline formation. The sequence of reactions is thought to involve citrulline formation in the small intestine using glutamine as a precursor (2). Citrulline is converted to arginine in the kidney (3). Classic work by Windmueller and Spaeth (4), measuring arteriovenous differences in citrulline (3.4 ± 0.7 μM) across a rat hindquarter speculated that skeletal muscle may make a quantitatively important contribution to whole body citrulline flux. Our laboratory has been extensively involved in studies of skeletal muscle protein and amino acid metabolism and citrulline has been seen on chromatographic separation of physiological media in which normal muscles had been incubated. There are reports of citrulline in exudates from wounded muscle (5) and from muscle cells isolated from wounds (6) but no reports in normal muscle, and no evidence of any kind bearing on the source(s) of its synthesis.

Several reactions can result in citrulline formation from arginine or glutamine:

1) Enzymes of the urea cycle. Arginine is converted to ornithine and urea by the action of arginase (EC 3.5.3.1), and ornithine is converted to citrulline by the action of ornithine transcarbamoylase (EC 2.1.3.3). When citrulline is formed from glutamine, the sequence of reactions also includes ornithine transcarbamoylase. The entire urea cycle is thought to be confined to the liver; however individual reactions are known to exist in other tissues. There is limited evidence for arginase activity (5-7), but no evidence for ornithine transcarbamoylase activity in skeletal muscle.

2) Arginine deiminases are a class of enzymes converting arginine to citrulline in a reaction where a guanido nitrogen is converted to ammonia. Arginine deiminases (EC 3.5.3.6) appear to be unique to single celled organisms (8), however peptidylarginine deiminase (EC 3.5.3.15) has been isolated from mammalian skeletal muscle (9,10). Protein-bound citrulline in skeletal muscle, derived from the activity of this enzyme, could be released as proteins are degraded.

3) Nitric oxide synthases (EC 1.14.23) are a group of related enzymes that convert arginine and molecular oxygen to citrulline, with concomitant formation of nitric oxide from one of the two chemically equivalent guanido nitrogen atoms. Nitric oxide synthase appears to have many isoforms, however, these may be generally classed as either: a) calcium-dependent, constitutive forms such as those found in the vasculature and brain, or b) calcium-independent forms inducible by endotoxin or cytokines, characteristic of mononuclear phagocytes (reviewed in 11-14). Since nitric oxide synthase activity was detected in cytosolic extracts of skeletal muscle by Salter *et al.* (15), and Nakane *et al.* (16) demonstrated the presence of nitric oxide synthase mRNA in the tissue, this enzyme appears to be a potential source of muscle citrulline. The studies described here were designed to measure citrulline production and to identify the origin of citrulline in skeletal muscle.

MATERIALS AND METHODS

Chemicals

L-arginine, L-citrulline, L-NMMA, L-NAME, lipopolysaccaride (*Escherichia coli* 055:B5, TCA extract), glucose, EGTA, HEPES, cycloheximide (*Streptomyces griseus*) and insulin (bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). TCA was purchased from Fisher Scientific (Fairlawn, N.J., U.S.A.). L-[*guanido*-^{14}C]arginine and L-[*ureido*-^{14}C]citrulline were purchased from Dupont New England Nuclear, (Mississauga, ON, Canada). [^{14}C]urea was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.). Ion exchange resins AG1-X8 (200-400 mesh) and analytical grade AG50W-X8 (200-400 mesh, H^+ form) were purchased from BioRad Laboratories

(Richmond, CA, U.S.A.). Hionic-flour™ and Soluene-350™ were purchased from Packard (Meriden, CT, U.S.A.). Scintillation cocktail Ecolite™ was purchased from ICN, (Costa Mesa, CA, U.S.A.).

In the cytosol experiments, chemicals were obtained from the following sources. NAPDH and tetrahydrobiopterin were purchased from Dr. Schircks Laboratories (Iona, Switzerland). L-NMMA was purchased from Wellcome Research (London, U.K.). [U-^{14}C]arginine was purchased from Amersham (Oakville, Ont. Canada). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals

Experiments were carried out in compliance with the guidelines of the Canadian Council of Animal Care. Male or female Sprague-Dawley rats of the Buffalo strain were obtained from a colony maintained in the department of Animal Science, University of Alberta. Rats were group housed in plastic bottomed cages and offered laboratory chow (Wayne Rodent Blox, Premier Lab Diets, Bartonville IL. U.S.A.) and water *ad libitum*. Rat rooms were held at constant humidity (80%) and temperature (24^{o}C). Lipopolysaccaride was prepared for injection by dilution (1 mg/ml) in sterile 0.9% NaCl and injected intraperitoneally at 3 mg/kg body weight 12 or 6 hr prior to experiments. Traumatized rats were subjected to a single controlled impact trauma to the medial aspect of the lower hind limb, using a blunt injury device, under halothane (Ayerst Laboratories, Montreal, Can.) anaesthesia as described by Fisher *et al.* (17). Injury was sustained primarily by the gastrocnemius muscle. Control rats were anaesthetized but not injured. Muscles were harvested 24 hr after trauma.

Muscle Incubation

Either epitrochlearis or thin longitudinal strips of gastrocnemius were used in the incubations. The epitrochlearis muscle is a thin tissue suited to incubation and metabolic studies *in vitro* (18). Strips of gastrocnemius were used in order to obtain samples of injured muscle. After rats were killed by CO_2 asphyxiation, muscles were rapidly dissected, weighed, and placed in a tube containing 3 ml incubation media in a shaking water bath (36^{o}C). After a 30 minute pre-incubation, muscles were transferred to 3 ml fresh media and incubated for another 3 hr. Unless otherwise noted preincubation media was the same as incubation media. Incubation media was supplemented Krebs-Ringer bicarbonate medium (pH 7.4, 119 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.25 mM NaH_2PO_2, 2.0 mM HEPES, 5.0 mM glucose and 0.5 U/ml insulin), oxygenated continously with a mixture of O_2 and CO_2 (19:1). Calcium free medium included 1 mM EGTA and excluded $CaCl_2$. Arginine and nitric oxide synthase inhibitors L-NMMA or L-NAME (19) were added as indicated in the tables and results. At the end of incubation, each muscle was placed in 1 ml of ice-cold 2% TCA, and the muscle and the media were frozen (-20^{o}C). Muscles were homogenized with a glass/glass homogenizer (room temperature) and rinsed with 1 ml of 2% TCA. The homogenate was allowed to stand at 4^{o}C for about 1 hr, then centrifuged at 1000 x *g* for 10 min. The TCA soluble fraction containing free amino acids in the intracellular and extracellular fluids was stored frozen until analyzed. When the TCA insoluble protein fraction was analyzed, it was washed twice with 1 ml of 2% TCA and then once with 1 ml of diethyl ether, and allowed to air dry.

High Performance Liquid Chromatography (HPLC)

Citrulline, arginine and tyrosine were measured in incubation media and TCA soluble tissue fraction by HPLC. The procedure was modified from Jones and Gilligan (20). Samples were mixed with the fluoraldehyde (o-phthaldialdehyde) reagent for derivatization before injection. Twenty-five µl of derivatized sample was injected onto a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with Supelco LC-18 reverse phase packing (20-40 µm). A Varian 5000 HPLC and Varian Fluorichrom detector (excitation 340 nm, emission 450 nm) were used to separate and measure the amino acids. Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom Chromatography data system. The two solvents used were 0.1 M sodium acetate and methanol. An internal standard, L-α-amino-β-guanidino proprionic acid, was added to each sample and used in the amino acid analyses. L-NAME was measured by comparing it to a concurrently run single point standard curve. The gradient began with 0%

methanol, 14% methanol at 0.1 min to 15% methanol at 22 min, from 22.1 to 26 min the methanol increased from 30 to 31%. At 26.1 min methanol was 47% and was increased to 50% at 38 min. The column was subsequently washed with 100% methanol for 2 min and then with 0.1 M sodium acetate for 5 min. Peaks were identified and quantified by comparison to a single point calibration curve determinined by running standards of known amino acids (including ornithine and taurine), with each group of samples. Blanks of incubation media were also run with each group of samples. Citrulline was clearly resolved from preceding threonine and succeeding arginine. The limit of detection of citrulline by HPLC (39 pmol/ml) was determined by analyzing serial dilutions of a standard solution.

Distinguishing Nitric Oxide Synthase from Arginase

To distinguish nitric oxide synthase activity from arginase activity, L-[*guanido*-^{14}C]arginine was used as a precursor. Nitric oxide synthase metabolism of this substrate yields [^{14}C]citrulline while arginase yields [^{14}C]urea (Fig. 5.1). [^{14}C]arginine was purified by ion exchange chromatography on AG1-X8 prior to use and added to incubation media at 0.18 μCi/ml for experiments presented in Table 5.3 and 0.11 μCi/ml for the experiment in Table 5.4. To separate [^{14}C]arginine and its products [^{14}C]urea and [^{14}C]citrulline from each other, the procedure of Gopalakrishna and Nagarajan (21) was modified. An AG 50W-X8(Na^+) column was mounted above a AG 50W-X8(H^+) column. Eluent amounts were modified as described below. After application of the sample (either incubation media or neutralized intracellular fraction) followed by 5 ml water the columns were separated. The Na^+ column was washed with 5 ml of 0.15 M (pH 6) sodium citrate hydrochloride, and then arginine was eluted with 10 ml of 0.5 M (pH 6.5) sodium citrate hydrochloride (first 2 ml were discarded). Columns were regenerated with 4 ml of 2 M NaOH. Urea was eluted from the H^+ column with 18 ml of water. The first 8 ml and the last 6 ml of water were discarded. The citrulline was eluted with 3 ml of 6 M HCl and the column was then washed with 2 ml of 2 M HCl. Both columns were then rinsed with water until the eluent was pH 6-7. Columns were reused up to 5 times.

Before samples were run through the columns, recoveries were tested with radiolabelled amino acids in supplemented KRB containing 1.0 mM arginine, 0.06 mM citrulline and 0.08 mM urea. [^{14}C]arginine was added at 0.18 μCi/ml KRB. [^{14}C]citrulline or [^{14}C]urea were added at 0.011 μCi/ml KRB. Very little arginine co-eluted in the citrulline or urea fractions (Table 4.1), but since arginine specific activity was high, the radioactivity due to arginine can approach that due to either citrulline or urea. Therefore for each experiment, backgrounds were established by running incubation media blanks (media that had been incubated without a muscle) through the columns. The average background was subtracted from the sample value to give actual citrulline or urea radioactivity. Average citrulline radioactivity was at least 300 dpm/3 hr greater than background. Radioactivity was measured by mixing aliquots of column eluent with a scintillation fluor (Ecolite™) and counting in a Packard 1600CA TriCard Liquid Scintillation analyzer. Total citrulline or urea production was calculated by dividing the citrulline or urea radioactivity (dpm) by TCA soluble arginine specific activity (dpm/nmol).

Hydrolysis of Muscle Protein

The amount of citrulline in muscle protein was determined after hydrolysis of homogenized, washed muscle protein. Approximately 45 mg of muscle protein was placed in 3 ml of 6 M HCl, the atmosphere sparged with N_2, and incubated at 110^{o}C for 24 hr. Steinert *et al.* (22) indicated that citrulline is converted to ornithine during acidic hydrolysis. To correct for this loss, a standard solution of citrulline and tyrosine was prepared and treated with 6 M HCl under the same conditions as for muscle protein hydrolysis, and incubated for various times from 0 to 30 hr. Linear regression analysis of citrulline concentration over time was significant (n=16, $p=9.09x10^{-7}$, r^2=83%), and at 24 hr 47% of the citrulline was lost. There was no significant loss of tyrosine (n=16, p=0.0954, r^2=19%). When the amount of citrullineand tyrosine in muscle protein were calculated (see below) correction factors of 47% and 0% respectively were used.

Determining Nitric Oxide Synthase Activity in Muscle Cytosol

Citrulline formation in muscle cytosol was determined using the method of Salter *et al.* (15). Briefly, muscles were frozen in liquid nitrogen, pulverized, and homogenized (Pro 200) at 4^{o}C in buffer

(1:4 w/v), (pH 7.2, 10 μM HEPES, 0.32 M sucrose, 0.1 μM EGTA, 1 mM DL-dithiotreitol, 10 μg/ml tryspin, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 100 μg/ml PMSF). After centrifugation (35 min @ 100,000 x g, 4°C) 20 μl of the intracellular fraction (supernatant) was added to 100 μl incubation media (pH 7.2, 50 mM KH_2PO_4, 0.24 mM $CaCl_2$, 1.2 mM $MgCl_2$, 60 mM valine, 1.2 mM citrulline, 22 μM arginine with 0.132 μCi/ml [U-^{14}C]arginine, 120 μM NADPH, 12 μM tetrahydrobiopterin) in the presence or absence of EGTA (1 mM) or EGTA and L-NMMA (1 mM each). For the dose response experiment, incubation media had varying levels of L-NMMA or L-NAME (Fig 5.2), with no EGTA. Cytosol was incubated for 12 min at 37°C. At the end of incubation the samples were moved to room temperature (20°C) and gently mixed with 1.5 ml of a slurry of 1:1 (v/v) Dowex 50W-X8, Na^+ form:water and then 4 ml of water. After the resin had settled, 3.5 ml of supernatant was collected, mixed with 14 ml of scintillation fluid (Ecolite™) and counted in a liquid scintillation counter. cNOS activity was calculated from the difference in radioactivity between incubation media with and without EGTA, divided by the calculated arginine specific activity of the buffer (20.7 dpm/pmol arginine). iNOS activity was calculated from the difference in radioactivity between incubation media with EGTA only, and incubation media with EGTA and L-NMMA. The remaining radioactivity in this system may be from impurities in the radiolabelled arginine, from trace amounts of arginine not bound to the resin, from citrulline synthesized by enzymes other than NOS or a combination of these three sources.

Statistical Analysis

Statistical analyses used were a multiway ANOVA, using rat as a source in the model and variation due to rat was removed. Three or more means which were significantly different were separated from each other by Least Significant Differences. The time course experiment was analyzed by repeated measures, and significantly different means (Period x Treatment) were separated by a T-test. Linear regression was used to analyze citrulline and tyrosine loss during acid hydrolysis. If $p<0.05$, results were considered significantly different. Pooled S.E.M. were calculated using Least Squares Means (SAS 6.06.01, SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Net Citrulline Release

Incubated intact epitrochlearis and medial gastrocnemius strips released citrulline into the incubation media. The net citrulline release ranged from 0.110 to 0.272 nmol/mg wet tissue/3 hr (Table 5.1). Citrulline release was not significantly affected by traumatic injury to the tissue (Table 5.1) or injection of animals with *E. coli* endotoxin (0.410 versus 0.417 ± 0.015 nmol/mg tissue/3 hr, $p = 0.997$). *In vitro* citrulline release was not affected by the addition of cycloheximide to the medium, but was attenuated by incubation in calcium-free medium (Table 5.1). The presence of added arginine (0.3 mM) or ornithine (0.05 mM) did not influence net citrulline release from the tissue.

Net tissue citrulline release was measured in the above experiments but these values do not incorporate any changes in the citrulline concentration in the tissue free amino acid pool (TCA soluble muscle homogenate). A decrease in the amount of free citrulline in muscle tissue over 3 hr of incubation was observed. Of the total released citrulline in the incubation media, 27-32% is due to citrulline leaking out of the muscle tissue. Therefore the data in Table 5.1 is an overestimate of citrulline synthesis. Since loss of tissue free citrulline did not vary with treatments (Table 5.2), treatments are comparable on the basis of net release.

Origin of Citrulline

The release of tyrosine from epitrochlearis muscles when protein synthesis was inhibited with cycloheximide was approximately threefold greater than that of citrulline (0.330 ± 0.019 and 0.117 ± 0.009 nmol/mg tissue/3 hr respectively, n=8). For a significant proportion of this citrulline to be derived from protein-bound citrulline, the ratio of tyrosine:citrulline in muscle protein would need to be in the order of 3:1. No detectable citrulline was found in epitrochlearis muscle protein hydrolysates (n=4) and considering the limits of detection of the assay, and citrulline degradation during acid hydrolysis, the

tyrosine:citrulline ratio of muscle protein is ≥ 48:1. Despite the presence of peptidylarginine deiminases in muscle (9), this would not appear to result in large amount of citrulline in a protein bound form.

Tissue metabolism of L-[*guanido*-^{14}C]Arginine

Skeletal muscle has a relatively large free pool of arginine (1.284 nmol/mg)(23). To test its equilibration with the media, 1.0 mM arginine (with L-[*guanido*-^{14}C]arginine at 400 dpm/nmol) was added to the incubation media at time zero. At 0.5 and 3.5 hours of incubation (36°C) the muscle free arginine specific activity was 259 ± 13 and 294 ± 13 dpm/nmol, (p=0.0923, n=8), indicating rapid equilibration between the incubation medium and the free intracellular pool. In a separate experiment muscle free arginine specific activity was not significantly different from media arginine specific activity (255 ± 12 and 225 ± 12 dpm/nmol respectively, p=0.0841, n=8). Release of labelled citrulline and urea into the media was also measured (Table 5.3).

Total citrulline and urea synthesis were measured by summing radioactivity in the TCA soluble fraction and in the media from muscles incubated in [^{14}C]arginine for the 3 hour incubation period. One of each muscle pair was incubated in a nitric oxide synthase inhibitor L-NAME (0.010 mM) which had no effect on citrulline synthesis (Table 5.3), as measured in TCA-soluble muscle homogenate and media.

L-NAME is a competitive inhibitor of NOS (19) and it is possible that the lack of inhibitor effect was due to a low dose of inhibitor, or too low an inhibitor:arginine ratio. Therefore the arginine concentration was decreased to 0.4 mM, while the calculated arginine specific activity (600 dpm/nmol) and the amount of inhibitor (2.0 mM) in the media were increased. Paired muscles were incubated in the presence or absence of the inhibitor for 9 hr, being transferred into fresh media every 3 hr. Due to problems in collecting the TCA soluble fractions, only products released into the media are reported. TCA soluble arginine specific activity was 470 ± 37 dpm/nmol at 9 hr (n=6), and the inhibitor had no effect of this variable ($p > 0.05$). Urea release was not influenced by L-NAME but did increase with time (Table 5.4). Labelled citrulline release increased with time (Table 5.4), and this increase was markedly reduced in the presence of the inhibitor between 6 and 9 hr, though there was no effect in the first 6 hr. The specific activity of citrulline in the media also increased with time, though it did not reach the levels of TCA soluble arginine specific activity. The inhibitor greatly reduced the citrulline specific activity and prevented a time-related increase. In none of the experiments did [^{14}C]citrulline release, determined by the use of the radioactive precursor, approach total citrulline release as measured by HPLC (Tables 5.3 and 5.4).

Inhibitor Transport

It was hypothesized that L-NAME was a poor inhibitor because it could not enter the cell. To test this hypothesis, muscles were incubated for 3 hr with 0.100 mM L-NAME and washed in ice-cold non-supplemented KRB three times for fifteen minutes each. The ice cold KRB washed out the extracellular space while preventing the amino acid transporters from transporting any arginine or L-NAME out of the cell. The arginine:L-NAME ratio in the TCA soluble muscle homogenate was measured. They were not significantly different (p=0.9031) in homogenates of washed or unwashed muscle (1.25 ± 0.05 or 1.20 ± 0.05 respectively), indicating that L-NAME enters the cell.

It was also hypothesized that L-NAME may not be as effective an inhibitor of NOS in muscles as in other tissues. To test this hypothesis, NOS activity in epitrochlearis muscle cytosol was measured (Table 5.5). The measured value for cNOS (0.136 ± 0.032 nmol/g tissue/min) is approximately 14% of that reported by Salter *et al.* (15), in unspecified rat skeletal muscle tissue. Both calculations assume that homogenized muscle cytosol had the same arginine concentration as the buffer, but muscle has a high level of intracellular arginine (1.24 nmol/mg)(23). When arginine concentration was measured in the homogenized muscle cytosol and used to calculate arginine specific activity, cNOS activity was 0.266 ± 0.073 nmol/g tissue/min. There was no measurable iNOS activity. Inhibitory dose response curves for both L-NMMA and L-NAME were measured in gastrocnemius muscle cytosol (Fig. 5.2). Both inhibitors reduce the amount of radioactivity to similar levels (>60%) in a concentration-dependant manner.

DISCUSSION

Synthesis of Citrulline and Urea

These experiments clearly demonstrated that skeletal muscle tissue synthesizes both citrulline and urea. Citrulline synthesis from muscle may contribute a large proportion of the body flux. Since the gastrocnemius and epitrochlearis have a fibre type distribution similar to several other muscles (24) the citrulline synthesis values from these muscles can be used as a reasonable estimate of citrulline synthesis by the whole body. In a 300 g rat, with 105 g muscle and an average citrulline synthesis between 7.7×10^{-5} and 19.0×10^{-5} μmol/mg/3 hr (citrulline release from Table 5.1 x 70% to account for intracellular citrulline release), rat skeletal muscle tissue would synthesize between 65 and 160 μmoles of citrulline per day, as much as the kidney normally converts to arginine daily (3). In comparison, the circulating plasma pool has only 0.533-0.564 μmoles of citrulline (0.052-0.055 μmole/ml plasma)(3,21). The cause of the variation in citrulline release is unknown at present, but multiple factors could be involved since there appear to be multiple sources of citrulline.

This is the first report of urea production (via arginase) in normal muscle tissue, though it has been reported in wounded muscle (5) and muscle cells cultured from damaged tissue (6). In rats, the amount of urea produced in muscle is tiny compared to liver production (66 μmol/day compared to 6.3×10^{6} μmole/day on a 15% protein diet, respectively)(25)

Source of Citrulline and Urea

There is little, if any, protein-bound citrulline in muscle despite the presence of peptidylarginine deiminase. Using enzymatic digestion of protein followed by descending paper chromatography, Nishimaki *et al.* (26) measured 1.37 μmol citrulline/g protein or 0.274 nmole/mg tissue in whole rabbit skeletal muscle. This value is approximately 7 times above the limit of citrulline detection using HPLC. Relative sensitivity of methods, or species, age or muscle differences are possible reasons why no citrulline was measured in rat but was measured in rabbit skeletal muscle. Even if rat muscles were to contain 0.274 nmol citrulline/mg tissue, for citrulline release from protein degradation to account for all the citrulline released would require the protein to turnover completely in 3 hr, highly unlikely in a tissue with protein half-lives estimated to vary from 1.3-180 days (27,28). Thus, citrulline release following degradation of muscle protein cannot account for all citrulline release measured.

The insensitivity of citrulline release to ornithine availability suggests that ornithine transcarbamoylase is not active in the muscle. This implies that citrulline is not produced either from glutamine or from arginine via urea cycle enzymes. The most likely enzyme to produce labelled citrulline from L-[*guanido*-^{14}C]arginine is nitric oxide synthase. Citrulline release was not affected by injecting rats with *E. coli* endotoxin, a stimulator of inducible nitric oxide synthase (12,14), with trauma, which brings mononuclear cells (presumably macrophages) into the area (17), or during incubation with cycloheximide, a protein synthesis inhibitor. These results suggest that the nitric oxide synthase in muscle is not an inducible isoform. The apparent increase in [^{14}C]citrulline release over 9 hr may be confounded by intracellular citrulline loss (Table 3.2), or may be due to an upregulation of cNOS as in cultured endothelial cells (29) or induction of iNOS. Citrulline release was sensitive to calcium, as is constitutive nitric oxide synthase. Salter *et al.* (15) also concluded that muscle tissue has cNOS but not iNOS, since there was no significant increase in citrulline production 6 hr after stimulation with endotoxin. Additional support for the presence of cNOS in skeletal muscle comes from Nakane *et al.* (16) who measured large amounts of brain-type cNOS mRNA and protein in skeletal muscle. In comparison cardiac muscle has cNOS (30) and expresses iNOS after exposure to cytokines while aortic smooth muscle has only iNOS (31) seen after exposure to cytokines.

Since significant amounts of citrulline do not appear to be formed via peptidylarginine deiminase or ornithine transcarbamoylase, and since arginine deiminase is not calcium dependant (32) it was concluded that the most likely source of citrulline was by the action of NOS. It was surprising to see that an inhibitor of NOS, L-NAME only altered citrulline production after long incubations at very high doses in comparison to those required in muscle cytosol. The experiment of washing the extracellular space demonstrated that L-NAME is able to cross the membrane, eliminating that as a possible reason for inhibitor inactivity. That L-NAME only prevented the increased citrulline release in the last 3 hr

incubation period and not in the first two periods may hint that L-NAME requires a long time to become active in muscle. It may be that the methyl esterase enzyme, which metabolizes L-NAME into its active form is less active in muscle than in other tissues were L-NAME has been used. In aortic rings, L-NAME is at least 10 x more potent than L-NMMA, though this difference is not seen in endothelial cell cytosol (19).

Labelled citrulline could also be produced by the activity of arginine deiminase, as suggested by Albina *et al.* (5). Arginine deiminase metabolizes free arginine to citrulline and ammonia (33). In skeletal muscle ammonia is also produced by amino acid or AMP deamination (25), so measuring ammonia production would not be specific for arginine deiminase activity. To date arginine deiminase has been described in several procaryotes (33), in protozoa and in unicellular green algae (cited in 8) but never in mammalian cells. Also, arginine deiminase does not require any cofactors, including calcium (32) so it is unlikely to be the source of calcium dependant citrulline production. It is possible that Albina *et al.* (5) were measuring the activity of nitric oxide synthase and not arginine deiminase.

The difference between labelled citrulline and total citrulline production that persists throughout a 9 hr incubation and the difference between the specific activity of intracellular arginine and media citrulline indicate that NOS is not the only source of citrulline production in muscle. It must be remembered that measurements of total citrulline release are confounded by citrulline loss from the intracellular pool. It has not been determined if these losses are sustained throughout the 9 hr incubation. Even if total citrulline is reduced by an estimated 27-32% (see page 34) it is still higher than labelled citrulline release. Further research will be required to determine the source of unlabelled citrulline.

Physiological Function

Both urea and ornithine are formed by arginase. Ornithine is a precursor of polyamines and arginase could be present in skeletal muscle to provide ornithine for the synthesis of ubiquitous polyamines (34).

Until muscle NOS can be inhibited, it is difficult to assess its physiological purpose. One physiological function of NOS is regulating vasodilation. Skeletal muscle pO_2 (35), arteriolar diameter (36,37), arterial pressure and regional blood flow (38) are sensitive to NOS inhibitors indicating NOS activity in skeletal muscle vasculature. Part of the NOS activity measured may be from the endothelial cells of muscle vasculature. Production of citrulline from cultured skeletal muscle cells (6) indicates that NOS may be in the muscle cell itself and not just in the surrounding tissues. Further research will discover the physiological functions of NOS in skeletal muscle.

Table 5.1. Net Citrulline Release from Incubated Skeletal Muscles

Muscles were incubated (3 hr) in supplemented KRB (see text) and citrulline release into the incubation media was measured by HPLC. Treatments are described in the table. Data are means ± pooled S.E.M. NA=not available.

	Rat Age (Weeks)	Rat Sex	Muscle	n	Treatment	Citrulline Release (nmol/mg tissue/3 hr)	Probability
Experiment 1	7-10	M	Gastrocnemius	12	control limb	0.225 ± 0.013	0.0706
				15	traumatized limb	0.191 ± 0.012	
Experiment 2	6	M	Gastrocnemius	7	1 mM Ca^{++}	0.210 ± 0.009	0.0008
				7	1 mM EGTA, 0 mM Ca^{++}	0.134 ± 0.009	
Experiment 3	NA	NA	Gastrocnemius	5	0.0 mM arginine	0.182 ± 0.006	0.0960
				5	0.3 mM arginine	0.163 ± 0.006	
Experiment 4	11	M	Epitrochlearis	8	control	0.110 ± 0.009	0.6490
				8	0.5 mM cycloheximide	0.117 ± 0.009	
Experiment 5	NA	NA	Epitrochlearis	6	0.000 mM ornithine	0.272 ± 0.020	0.0567
				6	0.050 mM ornithine	0.218 ± 0.020	

Table 5.2: Muscle Citrulline Content (TCA Soluble Citrulline)

TCA soluble citrulline was measured in pairs of muscles before and after a 3 hr incubation. In the first experiment 6 week old male rats had been injected with saline or *E. coli* endotoxin (3 mg/kg) 6 or 12 hr prior to the experiment. There was no significant difference in citrulline release between the two timepoints of *E. coli* endotoxin injection. In the next experiment, pairs of gastrocnemius muscle from 7 week old male, control rats were incubated in either 1 mM L-NMMA or 0.3 mM arginine. Data are means ± pooled S.E.M. Different superscripts within an experiment indicate that the means are significantly different ($p < 0.05$).

		TCA Soluble Citrulline (nmol/mg tissue)	
Muscle	n	Pre Incubation	Post Incubation
Epitrochlearis			
Saline injection	5	0.234 ± 0.017^{a}	0.115 ± 0.017^{b}
E. coli endotoxin injection	5	0.218 ± 0.017^{a}	0.105 ± 0.017^{b}
Gastrocnemius			
0.3 mM arginine	7	0.130 ± 0.011^{a}	0.051 ± 0.011^{b}
1.0 mM L-NMMA	7	0.111 ± 0.010^{a}	0.065 ± 0.010^{b}

Table 5.3: Metabolism of L-[*guanido*-^{14}C]Arginine

Epitrochlearis muscles were incubated in media containing 1 mM arginine labelled with [^{14}C]arginine (0.018 μCi/ml KRB) (described in text) in the presence or absence of 0.010 mM L-NAME. Release was calculated from radioactive products measured in the incubation media. Synthesis was calculated by summing radioactive products measured in the incubation media and in TCA soluble muscle homogenate. Labelled citrulline was calculated from citrulline radioactivity divided by intracellular arginine specific activity. Total citrulline was measured by HPLC (Total citrulline was not corrected for citrulline lost from the intracellular space, see Table 5.2). There are no significant differences in citrulline synthesis between muscles incubated with or without L-NAME (Analyzed using multiway ANOVA with rat as a block). N.D. = not determined. Data are means, n = 8.

	Release from Muscle (nmol/ mg tissue/ 3 hr)		
	Control	L-NAME	S.E.M.
Experiment 7 (11 week old female rats)			
Urea	0.020	N.D.	0.004
Labelled Citrulline	0.033	N.D.	0.005
Total Citrulline	0.120	N.D.	0.012
	Muscle Synthesis (nmol/ mg tissue/ 3 hr)		
	Control	L-NAME	Pooled S.E.M.
Experiment 8 (6 week old female rats)			
Urea	0.027	0.024	0.003
Labelled Citrulline	0.074	0.069	0.005
Total Citrulline	0.210	0.238	0.011

Table 5.4: Release of Citrulline and Urea from Incubated Skeletal Muscles with Time

Paired epitrochlearis muscles were incubated in the presence or absence of 2.0 mM L-NAME with [^{14}C]arginine (see text). Muscles were transferred to fresh media every 3 hr. Labelled citrulline was calculated from citrulline radioactivity divided by arginine specific activity. Total citrulline release was measured by HPLC (Total citrulline was not corrected for citrulline lost from the intracellular space, see Table 5.2). Data are means, n = 8. Different superscripts in rows and columns indicate significant differences ($p \leq 0.05$). Age and sex of rats is not available.

		Release from Muscle (nmol/mg tissue/3 hr)			
		Period (3 hr)			
		One	Two	Three	Pooled S.E.M.
Urea					
	Control	0.0039^{a}	0.0122^{ab}	0.0294^{b}	0.0060
	L-NAME	0.0019^{a}	0.0239^{b}	0.0293^{b}	0.0060
Labelled Citrulline					
	Control	0.0206^{a}	0.0725^{bd}	0.1410^{c}	0.0122
	L-NAME	0.0343^{a}	0.0938^{b}	0.0524^{ad}	0.0122
Total Citrulline					
	Control	0.1028^{a}	0.3284^{b}	0.2791^{b}	0.0449
	L-NAME	0.5341^{c}	1.2602^{d}	0.9749^{e}	0.0449
Media Citrulline Specific Activity (dpm/nmol)					
	Control	95.3^{b}	164.5^{c}	237.3^{d}	21.3
	L-NAME	33.3^{a}	36.3^{ab}	25.1^{a}	21.3

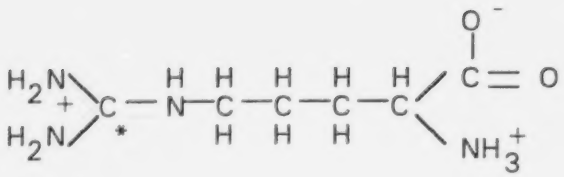
Table 5.5: Skeletal Muscle Cytosol Formation of Labelled Citrulline

Epitrochlearis muscle cytosol from 14 week old male rats was prepared (see text) and incubated for 12 min in the presence of 0, 1mM EGTA or 1 mM EGTA and 1 mM L-NMMA. The results are compared to those of Salter *et al.* (15) who used a similar method and also challenged rats with lipopolysaccaride 6 hrs prior to the experiment. Lipopolysaccaride made no significant difference in cNOS or iNOS activity.

	NOS Activity (nmole/g/min)	
	Peters	Salter *et al.*
n	4	3
cNOS	0.136 ± 0.032	0.94 ± 0.16
iNOS	0	0

Figure 5.1: Differentiating Citrulline Synthesis from Free Arginine via Urea Cycle Enzymes or NOS

If the urea cycle enzymes use [*guanido*-^{14}C]arginine as a substrate, the label (marked by an asterisk) will go into urea, and the citrulline subsequently formed from ornithine will be unlabelled. If citrulline is synthesized from arginine via NOS, the radiolabel will stay with the citrulline. Therefore labelled citrulline cannot come though the urea cycle enzymes.



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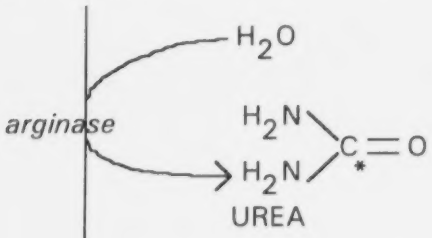

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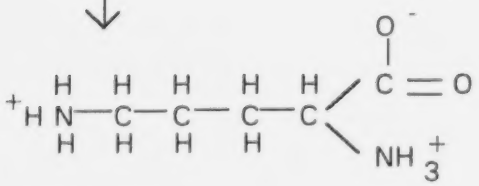

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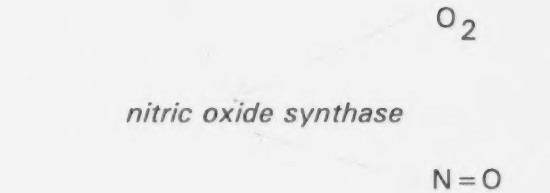
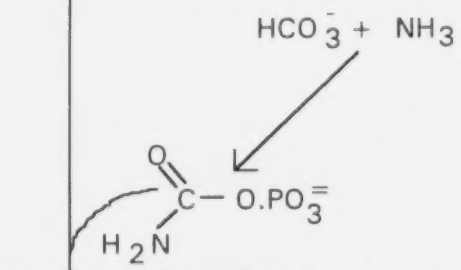
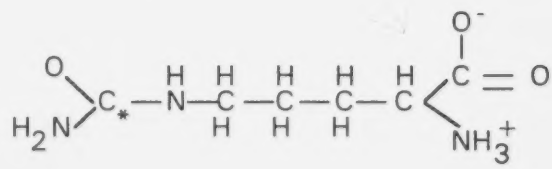
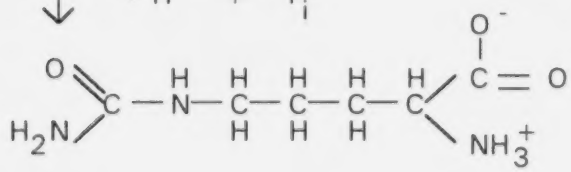






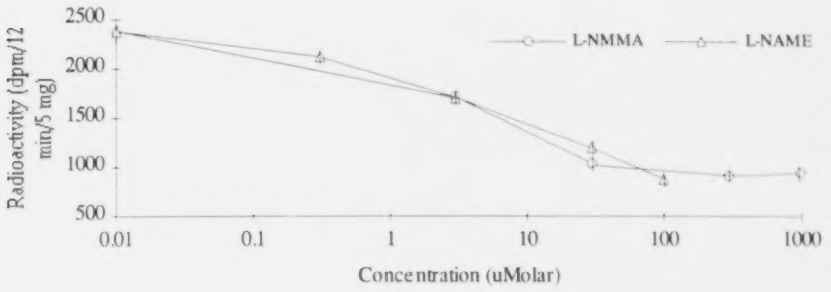
CITRULLINE



CITRULLINE

Figure 5.2: Skeletal Muscle Cytosol Formation of Labelled Citrulline in the Presence of Various Concentrations of L-NAME or L-NMMA

Gastrocnemius muscle cytosol from 14 week old male rats was prepared (see text) and incubated for 12 min in the presence or absence of several concentrations of L-NAME or L-NMMA. Data are means, n = 4, vertical bars indicate the S.E.M.



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VI. GENERAL DISCUSSION AND CONCLUSIONS

SUMMARY OF RESULTS

The objectives and hypotheses stated in Chapter 2 were tested as described in Chapters 3-5. The results are briefly summarized here. Measurements of citrulline release (Objectives 1 and 2) suggest that citrulline is not formed by the urea cycle or iNOS but by cNOS. It was determined that citrulline is lost from the intracellular pool during a 3 hr incubation of skeletal muscles, but that this loss is not affected by *in vivo* injection of enterotoxin or incubation with L-NMMA (Objective 3). Comparing tyrosine:citrulline ratios in incubation media and in hydrolysed muscle protein (Objective 4) suggests that protein-bound citrulline is not formed in rat skeletal muscle by the action of peptidylarginine deiminase. When [*guanido*-^{14}C]arginine was used as a substrate, radiolabelled citrulline and urea were formed (Objective 5) in whole muscle. Radiolabelled citrulline was also formed in muscle cytosol. Co-incubation with L-NAME reduced labelled citrulline formation, though high doses and long incubation periods were required for this to be seen in whole muscle incubations (Objective 6). A sensitive, direct measure of protein degradation was developed (Objective 7). The HPLC method to measure amino acids was successfully shortened (Objective 8), and it was determined that 47% of citrulline was lost during acid hydrolysis (Objective 9). The published method using cation exchange chromatography to separate arginine, urea and citrulline was successfully modified to work with physiological solutions (Objective 10) allowing a sensitive measure of both citrulline and urea synthesis.

Two lines of evidence agree with Hypothesis 1 that citrulline is synthesized in skeletal muscle. The first is that total citrulline release is more than citrulline lost from the intracellular pool, and secondly that radiolabelled citrulline is formed when muscles are incubated with radiolabelled arginine. This second line of evidence also indicates that arginine is a metabolic source of citrulline, as predicted by Hypothesis 2. Hypothesis 3, that nitric oxide synthase is active in skeletal muscle, is supported indirectly by the lack of evidence for citrulline synthesis either by urea cycle enzymes or by arginine deiminases. Direct support for Hypothesis 3 comes from the formation of labelled citrulline from [*guanido*-^{14}C]arginine and inhibition of citrulline formation by L-NAME. Because labelled citrulline formation never reaches the level of total citrulline formation, it is concluded that NOS is not the only source of citrulline formation in skeletal muscle. In addition, it was demonstrated that a small amount of urea is synthesized by whole muscle.

UNANSWERED QUESTIONS

The first question arises out of the inability to effectively inhibit the production of citrulline in intact muscle preparations compared with cytosol fractions. L-NMMA was not used as an inhibitor since it can also be metabolized into citrulline (1), confounding the interpretation of citrulline synthesis. L-NAME required long incubation times and high doses to be effective. L-NAME must be metabolized into the active inhibitor, N^G-nitro-arginine, by the removal of the methyl ester group. To test whether muscle can readily activate L-NAME, muscle incubations could be performed comparing L-NAME to N^G-nitro-arginine. N^G-nitro-arginine does not cross red blood cell membranes readily (2). If L-NAME does not cross muscle membranes readily it too may not be an effective inhibitor in muscle incubations. Current research continues to identify all the NOS isoforms and to develop isoform specific inhibitors. Further work on intact muscle could use these new inhibitors as they become available.

Another area that has not been explored is the role and fate of citrulline in the muscle. It has been assumed that citrulline itself is an innocuous by-product of NO production, rather than having a role itself. Nor has it been determined whether citrulline is metabolized in the muscle or exported, perhaps to the kidney for synthesis into arginine. Muscle incubations in radiolabelled citrulline and subsequent separation of possible metabolites (ie. arginine) would help to examine this question.

The third area that is not resolved is the contrast between the amounts of peptidylarginine deiminase found in skeletal muscle, and the small amount of citrulline found in muscle proteins. It would be worthwhile to compare the acid hydrolysis method used here with that of Nishimaki *et al.* (3) to compare sensitivities. As well as measuring citrulline in total muscle protein (1.37 ± 0.03 μmol/g),

Nishimaki *et al.* (3) divided muscle protein into several fractions and found a range of citrulline concentrations from <0.01 μmol/g protein in actin and myosin to 7.53 μmol/g protein in membrane associated proteins. It may be that the peptidlyarginine deiminases act on this relatively small portion of muscle cell protein.

Another perplexing question I raise is the source of unlabelled citrulline (Tables 5.3 and 5.4). All known metabolic sources of citrulline appear to have been ruled out. It may be that the methods used were not sensitive enough to detect citrulline from these pathways. iNOS and arginine deiminase would synthesize labelled rather than unlabelled citrulline from [*guanido*-^{14}C]arginine. Even if peptidylarginine deiminase was acting on proteins with a rapid enough turnover to release such large amounts of citrulline, after the first cycle remaining turnover would use labelled arginine for protein synthesis and hence labelled citrulline would be released. The urea cycle enzymes would produce unlabelled citrulline. It may be that ornithine transcarbamoylase activity is insensitive to the availability of ornithine or that ornithine does not cross muscle membrane, and in either case citrulline production would not vary with external ornithine concentration. Using labelled ornithine as a substrate would be a more sensitive way to measure ornithine transcarbamoylase activity in skeletal muscle. It is also possible that an alternative, yet unknown, pathway of citrulline synthesis exists.

PHYSIOLOGICAL FUNCTIONS OF NOS AND ARGINASE

Work in perfused hindquarters and muscle clearly indicate that NOS functions in the regulation of blood flow through the skeletal muscle (4,5). Presumably this NOS is located in the endothelium of the vessels. The neural cells of skeletal muscle nerves may also be synthesizing nitric oxide as a neurotransmitter. Resident macrophages or fibroblasts are present in small numbers in skeletal muscle. These cells are able to produce nitric oxide and citrulline (6). If these few cells produced large amounts of nitric oxide, they could account for muscle citrulline synthesis.

Citrulline is also produced by cultured muscle cells (7), evidence that myocytes themselves may contain NOS. Even if each myocyte produced only small quantities of nitric oxide, since myocytes are the most abundant cell in skeletal muscle, total citrulline and nitric oxide production would be significant. Since both NOS activation and skeletal muscle contraction are regulated by calcium availability, I speculate that muscle contraction and synthesis of nitric oxide are functionally related. In isolated frog skeletal muscle fibres, cGMP causes an earlier and higher peak height of calcium (8). Nitric oxide is known to stimulate cGMP production (9), so nitric oxide may regulate calcium movement. Alternatively since both cNOS and muscle contraction are stimulated by Ca^{++} it may be that the Ca^{++} influx triggers nitric oxide synthesis, then nitric oxide could move to the surrounding blood vessels to trigger vasodilation. This would parallel the situation in larger vessels where endothelial derived nitric oxide triggers smooth muscle relaxation and subsequent dilation of blood vessels (10). Vasodilation allows increased blood flow and consequently increased delivery of the nutrients required to support continued contraction. In cardiac papillary muscles cGMP analogues have a negative inotropic effect (11), indicating a possible role for nitric oxide in regulating contraction. Physically locating NOS in skeletal muscle (by using NOS antibodies on histological preparations) could help identify which cells contain NOS, and therefore possible functions of NOS but definitive work describing the physiological function of NOS in muscle awaits effective inhibition of the enzyme.

The reported work showed a small amount of arginase in the muscle. Ornithine is the first metabolic step towards synthesizing the ubiquitous, life-essential polyamines, therefore the role of arginase may be to provide ornithine, the substrate for polyamine synthesis. Arginase has been used to deplete arginine levels to reduce NOS effects in shock (12) but the relatively low level of arginase activity (0.027 nmole/mg tissue/3 hr, Table 5.3) compared to intracellular arginine levels (1.28 nmol/mg tissue)(13) would make it unlikely that arginase is used to regulate arginine levels in skeletal muscle.

REQUIREMENT FOR ARGININE

The discovery of NOS in skeletal muscle may help to quantify the semi-essential nature of arginine. When the body is in a stable state the kidney would be able to recycle enough citrulline,

formed either from small intestinal metabolism of glutamine, or NOS metabolism of arginine, to reform the required arginine. There would be no need for net input of raw products, since the citrulline and arginine would just cycle. In growing or ill animals with an increased demand for arginine in protein synthesis or for NOS activity an increase in substrate would be required. Averages of figures published in Salter *et al.* (14) show an increase of citrulline production from 0.59 nmol/min/g tissue (cNOS) to 1.51 nmol/min/g tissue (cNOS plus iNOS) after injection with endotoxin, a three fold increase in arginine use. If the body is unable to synthesize the additional arginine it must be provided from dietary sources.

CELLULAR PROTECTION FROM NITRIC OXIDE

The widespread activity and potential cytotoxicity of nitric oxide means that cells synthesizing nitric oxide must also regulate NOS activity to prevent undesired effects. The first defence against nitric oxide is its short half life (≤5 sec)(15), preventing any build-up of the free radical. The second defense is that nitric oxide readily permeates membranes (16) so it will disperse in all directions, again reducing concentrations in any single location of the cell. Scavengers of nitric oxide including oxygen radicals and heme containing proteins like hemoglobin or myoglobin (16) also decrease nitric oxide concentrations. Since the picomolar amounts of nitric oxide released by cNOS have reversible effects on enzymes and the concentrations of nitric oxide drops rapidly, there are few deleterious effects of nitric oxide produced by cNOS. All these mechanisms also work to protect the cells against the lethal nanomolar amounts of nitric oxide produced by iNOS. Since only cytokine activated macrophages produce nitric oxide, there is target for the nitric oxide produced. In addition, cytokine-activated macrophages also produce arginase (6), which will reduce the amount of arginine available for nitric oxide synthesis, reactive oxygen intermediates (6) which will scavenge nitric oxide, and TGFβ (6) which will suppress NOS activity. Therefore several mechanisms exist to regulate the amount of nitric oxide in the tissue.

CONCLUSION

The described experiments have established that arginine is metabolized into citrulline in skeletal muscle and that cNOS is the enzyme most likely to be responsible. Further research is required to identify the NOS isoform and to determine if it has a physiological role other than vasodilation in normal and stressed muscle. Understanding the complete role of NOS in skeletal muscle will allow the treatment of muscle dysfunctions. It will also identify possible skeletal muscle complications to using nitric oxide inhibitors in the whole body.

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VII. APPENDIX 1: DETAILS OF METHODS FOR MEASURING CITRULLINE

HPLC ANALYSIS OF CITRULLINE: REAGENT, SAMPLE AND STANDARD PREPARATION

Reagent Solutions

All reagents, including water were of HPLC grade.

Reagents

Solvent A: 27.2 g NaOH was dissolved in ~1.6 l of water. 1.5 M NaOH or acetic acid were used to adjust the solution to pH 7.2. 180 ml methanol and 10 ml tetrahydrofuran were added and the solution was stirred with the lid on to avoid unpleasant odours. Volume was brought to 2 l with water. Stable for two weeks.

Solvent B: Methanol

Fluoraldehyde Reagent: 0.50 g of o-phthaldialdehyde was dissolved in 12.5 ml methanol and 112 ml 0.04 M sodium borate buffer (pH 9.5, 15.25 g sodium borate in 1 litre water), 0.50 ml mercaptoethanol, and 4 ml Brij 35™ were added. This solution was freshly made each week.

Sample Buffer: 1.2 g benzoic acid was added to 25 ml of saturated $K_2B_4O_7$ and brought to 100 ml with water. Stable at 4 ^{o}C for months.

Internal Standard Solutions

Stock solution: 0.0913 g L-α-amino-β-guanidino proprionic acid was dissolved in 100 ml water (5 mol/ml). Stable at 4 ^{o}C for months.

Working solution: 0.05 ml AGPA stock into 25 ml water. This solution was made fresh daily.

Amino Acid Solutions

Citrulline stock solution: 21.7 g citrulline was dissolved in 25 ml water (4.95 mol/ml). Stable at 4^{o}C for months.

Amino Acid Working Solution: 0.100 ml amino acid stock solution (2.5 μmol/ml, Sigma) and 0.05 ml citrulline stock were added to 25 ml water or other solvent (see sample preparation). This solution was made fresh daily.

Preparation of Standards and Samples

Samples in Incubation Media

Amino acid standard working solution was made up in water.

Standard vials

0.05 ml working amino acid standard (A.A. STD)
0.05 ml working internal standard (I.S. STD)
0.05 ml sample buffer
0.55 ml water

Sample vials

0.25 ml sample
0.05 ml I.S. STD
0.05 ml sample buffer
0.35 ml water

Samples in TCA

Amino acid standard working solution was made up in TCA of the same concentration as the sample TCA.

Standard vials

0.05 ml A.A. STD
0.10 ml I.S. STD
0.05 ml saturated $K_2B_4O_7$
0.20 ml water

Sample vials

0.10 ml sample
0.10 ml I.S. STD
0.05 ml saturated $K_2B_4O_7$
0.15 ml water

Samples in 6 M HCl

Amino acid standard working solution was made up in 6 M HCl. One ml of working solution was placed in a borosilicate tube. The standard and all samples were made basic with 12 M NaOH (approximately 0.4 ml NaOH/ ml sample). Basic amino acid standard and samples were used to make up standard vials.

Standard vials

0.10 ml A.A. STD
0.10 ml I.S. STD
0.40 ml saturated $K_2B_4O_7$
0.15 ml water

Sample vials

0.25 ml sample
0.10 ml I.S. STD
0.40 ml saturated $K_2B_4O_7$
0.00 ml water

Samples in 0.5 M Sodium Citrate

Amino acid standard working solution was made up in 0.5 M sodium citrate.

Standard vials

0.10 ml A.A. STD
0.10 ml I.S. STD
0.10 ml sample buffer
0.40 ml water

Sample vials

0.10 ml sample
0.10 ml I.S. STD
0.10 ml sample buffer
0.40 ml water

REAGENTS FOR CATION EXCHANGE METHOD

All reagents were laboratory grade. Water was distilled and deionized.

2 M NaOH:	8 g NaOH into 100 ml of water.
2 M HCl:	20 ml of concentrated HCl into 70 ml water, brought to 100 ml with water.
6 M HCl:	60 ml of concentrated HCl into 30 ml water, brought to 100 ml with water.
0.15 M Sodium Citrate HCl:	8.82 g of Trisodium citrate into about 180 ml water. Adjusted to pH 6 with 6 M HCl, brought to 200 ml with water.
0.50 M Sodium Citrate HCl:	29 g of Trisodium citrate into about 180 ml water. Adjusted to pH 6.5 with 6 M HCl, brought to 200 ml with water.
5 N KOH:	28 g of KOH into 100 ml of water

Table A2.1: Skeletal Muscle Cytosol Formation of Labelled Citrulline in the Presence of Various Concentrations of L-NAME or L-NMMA

Gastrocnemius muscle cytosol from 14 week old male rats was prepared (see Chapter V) and incubated for 12 min in the presence or absence of several concentrations of L-NAME or L-NMMA. At 1000 μM L-NMMA or 100 μM L-NAME it was assumed that all NOS was inhibited, and that citrulline production was at baseline. Data are means, n=4, ND=Not determined. This data is also presented in Figure 5.2.

Inhibitor Concentration (μM)	Citrulline Formation (pmol/mg/min)	
	L-NMMA	L-NAME
0.0	2.396 ± 0.559	2.330 ± 0.510
0.3	ND	1.971 ± 0.516
3.0	1.330 ± 0.304	1.299 ± 0.307
30.0	0.229 ± 0.109	0.531 ± 0.423
300.0	0.120 ± 0.076	ND